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Binding and effect of K_{ATP} channel openers in the absence of Mg^{2+}

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1 Openers of ATP-sensitive K^+ channels (K_{ATP} channels) are thought to act by enhancing the ATPase activity of sulphonylurea receptors (SURs), the regulatory channel subunits. At higher concentrations, some openers activate K_{ATP} channels also in the absence of MgATP. Here, we describe binding and effect of structurally diverse openers in the absence of Mg^{2+} and presence of EDTA.

2 Binding of openers to SUR2B was measured using a mutant with high affinity for [^{3}H]glibenclamide ([^{3}H]GBC). In the absence of Mg^{2+}, 'typical' openers (benzopyrans, cyanoguanidines and aprikalim) inhibited [^{3}H]GBC binding with K_i values $\sim 200\times$ higher than in the presence of MgATP. Minoxidil sulphate and nicorandil were inactive, whereas binding of diazoxide was unaffected by MgATP.

3 In the absence/presence of MgATP, *N*-cyano-*N*′-(1,1-dimethylpropyl)-*N*″-3-pyridylguanidine (P1075) activated the Kir6.2/SUR2B channel in inside–out patches with $EC_{50} = 2000/67$ nM and $E_{max} = 32/134\%$. In the absence of Mg^{2+}, responses were variable with only a small part of the variability being explained by a decrease in channel responsiveness with time after patch excision and to differences in the ATP sensitivity between patches.

4 The rank order of efficacy of the openers was P1075 > rilmakalim ~ nicorandil > diazoxide > minoxidil sulphate.

5 The data show that structurally diverse openers are able to bind to, and to activate the Kir6.2/SUR2B channel by a pathway independent of ATP hydrolysis. These effects are observed at concentrations used to define the biochemical mechanism of the openers in the presence of MgATP and allow the openers to be classified into 'typical' and 'atypical' KCOs with diazoxide standing apart.
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Keywords: K_{ATP} channel openers (KCOs); mutant sulphonylurea receptor SUR2B(Y1206S); levcromakalim; rilmakalim; P1075; pinacidil; aprikalim; diazoxide; minoxidil sulphate; nicorandil

Abbreviations: GBC, glibenclamide; HEK cells, human embryonic kidney 293 cells; K_{ATP} channel, ATP-sensitive K^+ channel; KCOs, K_{ATP} channel openers; Kir, inwardly rectifying K^+ channel; NBD, nucleotide binding domain; P1075, *N*-cyano-*N*′-(1,1-dimethylpropyl)-*N*″-3-pyridylguanidine; SUR, sulphonylurea receptor

Introduction

ATP-sensitive K^+ channels (K_{ATP} channels) are closed by intracellular ATP and opened by MgADP; hence, they link the metabolic state of the cell to membrane potential and excitability (Ashcroft & Ashcroft, 1990). These channels are the target of the hypoglycaemic sulphonylureas like glibenclamide (GBC) which induce channel closure, and of the K_{ATP} channel openers (KCOs). The openers form a structurally heterogeneous class of compounds, which activate the channel primarily in vascular smooth muscle, thereby inducing hypotension (Ashcroft & Ashcroft, 1990; Edwards & Weston, 1994). Major representatives of the KCOs are the cyanoguanidines such as pinacidil and *N*-cyano-*N*′-(1,1-dimethylpropyl)-*N*″-3-pridylguanidine (P1075), the benzopyrans such as levcromakalim, and rilmakalim and the thioformamides like aprikalim; in addition, there are special cases like minoxidil sulphate, nicorandil and diazoxide (see Figure 1 for structures). New openers are being developed as therapies for asthma and cardiac ischaemia (benzopyrans, Buchheit *et al*., 2002; Grover *et al*., 2002) and irritable bladder (dihydropyridines, Gopalakrishnan *et al*., 2002).

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K_{ATP} channels are hetero-octameric complexes of pore-forming subunits, Kir6.x, and of regulatory subunits, the sulphonylurea receptors (SURs) with the subunits being arranged in a 1 : 1 stoichiometry $(Kir6.x/SUR)_4$ (Clement IV *et al*., 1997; Shyng & Nichols, 1997; for a review see Gonoi & Seino, 2000). Of both Kir6.x and SUR, subtypes exist. Most K_{ATP} channels contain Kir6.2. In these channels, inhibition by ATP is mediated by ATP binding to Kir6.2 (Tucker *et al*., 1997), whereas channel activation by MgADP is conferred by SUR (Nichols *et al*., 1996; Gribble *et al*., 1998). SURs are members of the ATP-binding cassette proteins with two nucleotide binding domains (NBDs), of which NBD2 in particular possesses ATPase activity (Bienengraeber *et al*., 2000; Matsuo *et al*., 2000). In addition, SUR is endowed with the binding sites for the sulphonylureas and the KCOs (Aguilar-Bryan *et al*., 1995; Sakura *et al*., 1995; Hambrock *et al*., 1998; Schwanstecher *et al*., 1998). Of the SUR subtypes, SUR1 is expressed predominantly in neurons and in pancreatic β cells, and SUR2 in muscle cells with SUR2A in skeletal and cardiac myocytes and SUR2B in smooth muscle cells.

Radioligand binding studies have shown that high-affinity binding of KCOs to native K_{ATP} channels and to recombinant SUR requires the presence of both Mg^{2+} and ATP (or other

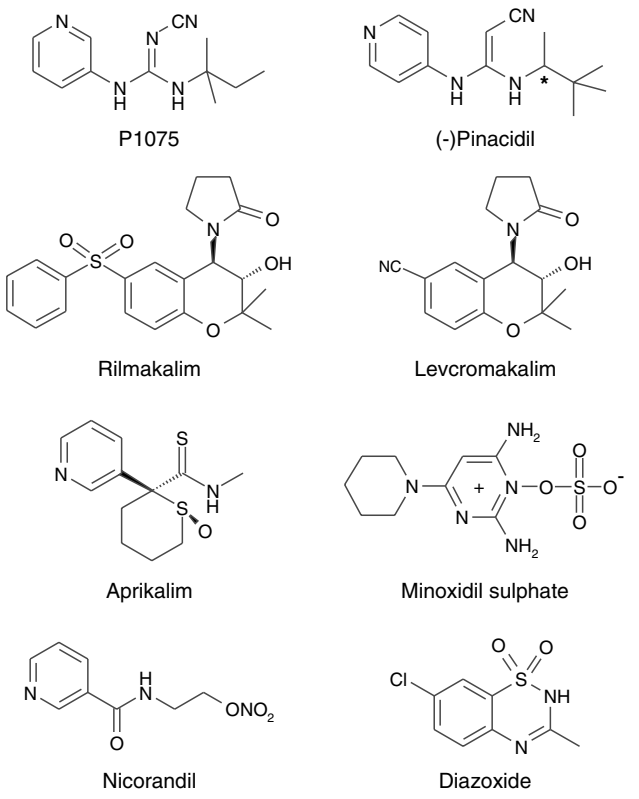


Figure 1 Structures of the KCOs used in this study. The cyanoguanidines are exemplified by P1075 and the active enantiomer of pinacidil (*R*, (−)), the benzopyrans by rilmakalim and levcromkalim (both active enantiomers, conformation (3*S*,4*R*)) and the thioformamides by aprikalim (1*R*,2*R* (−)).

hydrolysable nucleotides; Niki & Ashcroft, 1991; Schwanstecher *et al*., 1992; Quast *et al*., 1993; Dickinson *et al*., 1997; Hambrock *et al*., 1998; Schwanstecher *et al*., 1998), and it was suggested that ATP hydrolysis was required to bring SUR into a conformation with high affinity for the openers (Schwanstecher *et al*., 1998). Moreover, the KCOs increase the ATPase activity of SUR thereby increasing the local concentration of MgADP, the physiological opener of the channel (Bienengraeber *et al*., 2000). In addition, Zingman *et al*. (2001) have shown that pinacidil stabilised SUR in a MgADP-bound posthydrolytic conformation.

On the other hand, there is ample evidence that KCOs are able to activate SUR2 containing K_{ATP} channels also in the absence of MgATP, that is, by a pathway independent of ATP hydrolysis (skeletal muscle: Allard & Lazdunski, 1993; Forestier *et al*., 1993, 1996; Kir6.2/SUR2A: D'hahan *et al*., 1999a; Babenko *et al*., 2000; Gribble *et al*., 2000; Kir6.2/SUR2B: Reimann *et al*., 2000). In these studies, $\sim 10\times$ higher opener concentrations were used than employed usually in the presence of MgATP; however, precise concentration dependencies are lacking. MgATP slows down the rate of channel closing upon washout of the opener, suggesting that it slows dissociation of the opener from the channel (Gribble *et al*., 2000; Reimann *et al*., 2000, 2001). With the proviso that the association kinetics is not much affected, this means that MgATP increases the affinity of SUR2 for the opener, a conclusion reached also by the binding studies mentioned above. However, with the exception of P1075 (Löffler-Walz *et al*., 2002), binding of openers to SUR2 in the absence of MgATP has not yet been examined.

It was the aim of this study to examine the ability of structurally different KCOs (Figure 1) to bind to SUR2B and to activate the Kir6.2/SUR2B channel in the absence of MgATP. Thereby, we wanted (i) to determine the relation between binding and channel opening *via* the pathway independent of ATP hydrolysis and (ii) to see whether there were differences between the structurally different KCOs. Of the SUR subtypes, SUR2B was chosen since it has the highest affinity for the KCOs (with the exception of diazoxide) (Schwanstecher *et al*., 1998; Hambrock *et al*., 1999); the Kir6.2/SUR2B channel was chosen since it is more stable in inside – out patches than the Kir6.1/SUR2B channel (Yamada *et al*., 1997; Satoh *et al*., 1998). Binding experiments in the absence of MgATP were performed using [^{3}H]GBC as the radioligand, which shows increased binding to SUR2B in the absence of MgATP (Löffler-Walz *et al*., 2002). However, even then, the affinity of SUR2B for GBC ($K_D = 22$ nM) is not quite sufficient to obtain good-quality binding data (Löffler-Walz *et al*., 2002). Use was therefore made of a point mutation in SUR2B, in which tyrosine 1206 (mouse numbering) is replaced by serine, the corresponding amino acid in SUR1 (Ashfield *et al*., 1999) and which results in a higher affinity of SUR2B for GBC (~ 4 nM) (Toman *et al*., 2000; Hambrock *et al*., 2001).

Methods

Cell culture and transfection

SUR2B(Y1206S) was constructed from murine SUR2B (GenBank D86038, Isomoto *et al*., 1996) using the QuikChange Site-Directed Mutagenesis System (Stratagene, Amsterdam, The Netherlands) as described (Hambrock *et al*., 2001). Human embryonic kidney (HEK) 293 cells were cultured in minimum essential medium containing glutamine and supplemented with 10% fetal bovine serum and 20 μg ml^{-1} gentamycin (Hambrock *et al*., 1998). Cells were transfected with the mammalian expression vector pcDNA3.1 (Invitrogen, Karlsruhe, Germany) containing the coding sequence of murine SUR2B or SUR2B(Y1206S) using lipofectAMINE and OPTIMEM (Invitrogen), and cell lines stably expressing these proteins were generated as described (Hambrock *et al*., 1998). Cells transiently coexpressing SUR2B (wild-type) and murine Kir6.2 (D50581, Inagaki *et al*., 1996) were transfected with the plasmids at a molar ratio of 1 : 1. In cotransfections prepared for electrophysiological experiments, the pEGFP-C1 vector (Clontech, Palo Alto, CA, U.S.A.), encoding for green fluorescent protein, was added for easy identification of transfected cells. At 2 – 4 days after transfection, cells were used for binding studies and electrophysiological experiments.

Membrane preparation and radioligand binding competition experiments

For cells stably expressing SUR alone, the antibiotic was withdrawn from the culture medium 3 days prior to membrane preparation. Membranes were prepared as described (Hambrock *et al*., 1998). Protein concentration was determined according to Lowry *et al*. (1951) using bovine serum albumin as the standard.

For radioligand binding competition experiments in the absence/presence of MgATP, membranes were added to an incubation buffer containing (in mM): HEPES, 5; NaCl, 139; KCl, 5; $MgCl_2$, 0/2.2; EDTA, 1/0, and Na_2ATP 0/1, and supplemented with the radioligand ([^{3}H]GBC ~2.5 nM or [^{3}H]P1075 ~1.5 nM) and the inhibitor of interest. After equilibrium was reached (15 min for [^{3}H]GBC and 25 min for [^{3}H]P1075), incubation was stopped by diluting 0.3 ml aliquots (in triplicate) in 8 ml of ice-cold quench solution (50 mM Tris-(hydroxymethyl)-aminomethane, 154 mM NaCl, pH 7.4). Bound and free ligands were separated by rapid filtration over Whatman GF/B filters, washed twice with quench solution and counted for [^{3}H] in the presence of 6 ml of scintillant (Ultima Gold: Packard, Meriden, CT, U.S.A.). Nonspecific binding (B_{NS}) of [^{3}H]GBC/[^{3}H]P1075 was determined in the presence of 100/10 μM P1075 (Hambrock *et al*., 2001) and was ~25/10% of total binding.

Electrophysiological experiments

The patch-clamp technique was used in the inside – out configuration as described by Hamill *et al*. (1981). Patch pipettes were drawn from borosilicate glass capillaries (GC 150 T, 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; HEPES, 10; titrated to pH 7.4 with NaOH at 22°C. Filled 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^+ – EDTA – Ringer solution containing (in mM) KCl, 140; EDTA, 5; D(+)-glucose, 11; HEPES, 10; titrated to pH 7.2 with NaOH at 22°C. For experiments in the presence of Mg^{2+}, the pipe solution contained KCl, 143; $CaCl_2$, 1; D(+)-glucose, 11; HEPES, 10; EGTA, 5; $MgCl_2$ was added such that $[Mg^{2+}]_{free}$ was 0.7 mM. ATP was prepared as 100 mM stock solution and added as indicated. Openers were dissolved as described below and added to the pipe solution. Patches were clamped to −50 mV.

Data were recorded with an EPC 9 amplifier (HEKA, Lambrecht, Germany) using the 'Pulse' software (HEKA). Signals were filtered at 200 Hz using the four-pole Bessel filter of the EPC9 amplifier and sampled with 1 kHz.

Data analysis

Binding equilibrium inhibition curves were analysed using the logarithmic form of the Hill equation

$$y = 100 - A(1 + 10^{n(px - pIC_{50})})^{-1} \quad (1)$$

Here A denotes the extent (amplitude) of inhibition, $n(=n_H)$ the Hill coefficient and IC_{50} the midpoint of the curve with $pIC_{50} = -\log IC_{50}$; x is the concentration of the compound under study with $px = -\log x$. If necessary, two-component analysis was used with $n_H = 1$. IC_{50} values were converted into inhibition constants, K_i, by correcting for the presence of the radioligand, L, according to the equation

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

where K_D is the equilibrium dissociation constant of the radioligand. In case of homologous competition experiments, the inhibition constant K_i is identical to the K_D value. In general, the correction was between 1.5 and 2.0.

The concentration dependence of channel opening was analysed using the ascending form of the Hill equation and taking all individual data points into account. Some electrophysiological data were modelled by fitting the exponential function for decay or growth in the form of

$$y = a\exp(-kx) + b \text{ (decay) or}$$
$$y = a(1 - \exp(-kx)) \text{ (growth)} \quad (3)$$

Data are shown as means ± s.e.m. Fits of the equations to the data were performed according to the method of least squares using the programme SigmaPlot 6.1 (SPSS Science, Chicago, IL, U.S.A.). Individual binding experiments were analysed and the parameters averaged assuming that amplitudes and pIC_{50} values are normally distributed (Christopoulos, 1998). In the text, IC_{50} values are given followed by the 95% confidence interval in parentheses. In calculations involving two mean values with standard errors, propagation of errors was taken into account according to Bevington (1969). Significance of differences between two (normally distributed) parameters was assessed using the two-tailed unpaired Student's t-test.

Materials and solutions

[^{3}H]P1075 (specific activity 4.5 TBq (117 Ci) $mmol^{-1}$) was purchased from Amersham Buchler (Braunschweig, Germany) and [^{3}H]GBC (specific activity 1.85 TBq (50 Ci) $mmol^{-1}$) from Perkin-Elmer Life Sciences (Bad Homburg, Germany). The reagents and media used for cell culture and transfection were from Invitrogen. Na_2ATP was from Roche Diagnostics (Mannheim, Germany) and GBC from Sigma (Deisenhofen, Germany). The following drugs were kind gifts of the pharmaceutical companies indicated in parentheses: P1075 (Leo Pharmaceuticals, Ballerup, Denmark), levcromakalim (GlaxoSmithKline, Harlow, U.K.), rilmakalim (Aventis, Frankfurt, Germany), aprikalim (Aventis, Paris, France), nicorandil (Merck, Darmstadt, Germany) and diazoxide (Essex Pharma, München, Germany). Minoxidil sulphate and (−)pinacidil were synthesised by Dr P.W. Manley (Novartis, Basel, Switzerland). K_{ATP} channel modulators were dissolved in dimethyl sulphoxide/ethanol (50/50, v v^{-1}) to give stock solutions of 0.1 (most KCOs) to 0.01 M (diazoxide). These were further diluted with the same solvent or with incubation buffer to give final solvent concentrations <0.3% with the exception of diazoxide (3% solvent in all binding experiments).

Mg^{2+}-free solutions (no Mg^{2+} added, contaminating Mg^{2+} ≤10 μM (Forestier & Vivaudou), 1993, EDTA = 1 or 5 mM) contained ≤10 nM $[Mg^{2+}]_{free}$ (calculated as described in Hambrock *et al*., 1998).

Results

Opener binding to SUR2B(Y1206S)

First, we wanted to examine to which degree the mutation Y1206S in SUR2B affected opener binding; in case of P1075, only a small decrease in K_D by less than a factor of 2 was

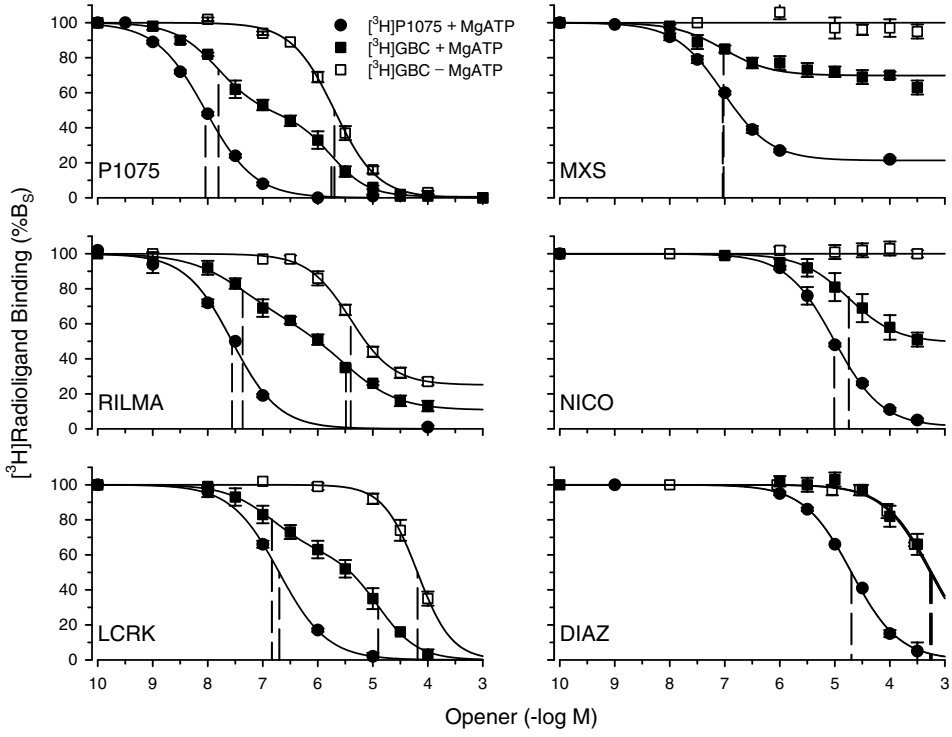


Figure 2 Binding of KCOs to SUR2B(Y1206S). Competition curves with [^{3}H]P1075 in the presence of 1 mM MgATP and with [^{3}H]glibenclamide ([^{3}H]GBC) in the presence and absence of MgATP (1 mM) are shown. pIC_{50} values are indicated by vertical lines. RILMA, rilmakalim; LCRK, levcromakalim; MXS, minoxidil sulphate; NICO, nicorandil: DIAZ, diazoxide. Data are expressed as specific binding (percentage of B_S); $n = 3-5$ and fitting curves were calculated as described in Methods.

observed (Hambrock *et al*., 2001). Therefore, the opener binding properties of SUR2B(Y1206S) were studied in the [^{3}H]P1075 competition assay using KCOs selected from different structural groups (Figure 1); these experiments were conducted in the presence of MgATP (1 mM). Figure 2 shows some of the inhibition curves; the results of all KCOs included in the study are summarised in Table 1. Inhibition curves were regular with the Hill coefficients close to 1 and generally reached completion; the exception was minoxidil sulphate which inhibited [^{3}H]P1075 binding by only 79 ± 1%. These results were compared with those obtained earlier with the wild-type (Table 1). Correlation analysis using the respective pK_i values gave a correlation line ($r^2 = 0.98$) parallel to but separated from the line of identity by 0.13 ± 0.05 log units (not illustrated). This means that, on average, the K_i values for the mutant were 1.4 (95% confidence interval (1.0, 1.8)) times higher than those for wild-type SUR2B.

Table 1 Binding of KCOs to SUR2B(Y1206S) and SUR2B wild-type as seen in [^{3}H]P1075 competition assays in the presence of MgATP (1 mM)

Opener	*SUR2B(Y1206S)* K_i *(nM)*	*SUR2B*[a] K_i *(nM)*
P1075	6.5 (6.2, 6.8)	4.4 (3.5, 5.5)
(−)Pinacidil	46 (42, 50)	23 (21, 26)
Rilmakalim	20 (15, 26)	21 (18, 26)
Levcromakalim	148 (141, 155)	115 (110, 120)
Aprikalim	437 (417, 457)	324 (224, 468)
Diazoxide	17,000 (16,000, 18,000)	7800 (5900, 10,000)
Minoxidil sulphate[b]	72 (63, 83)	48 (38, 60)
Nicorandil	7100 (6500, 7800)	9100 (6900, 12,000)

The Hill coefficients were not significantly different from unity and, with the exception of minoxidil sulphate, the amplitudes of the inhibition curves were 100% B_S. For conditions, see Figure 2. [a]With the exception of rilmakalim, data for SUR2B are from Hambrock *et al.* (1998; 1999). [b]Amplitudes were 79 ± 1 and 72 ± 2% B_S for SUR2B(Y1206S) and wild-type, respectively.

[^{3}H]GBC – opener competition curves

[^{3}H]GBC binding to SUR is reduced by MgATP; in case of wild-type and mutant SUR2B this was because of a decrease in the number of binding sites by 75%, whereas the affinity remained unchanged ($K_D \sim 22$ and 4 nM for wild-type and mutant SUR2B, respectively; Löffler-Walz *et al*., 2002; Hambrock *et al*., 2002). In the presence of MgATP, the [^{3}H]GBC – opener inhibition curves for P1075, (−)pinacidil, rilmakalim, levcromakalim and aprikalim were biphasic (Table 2, Figure 2). The high-affinity component comprised $\sim 40-50$% of the total amplitude with K_i values close to those determined in the [^{3}H]P1075 experiments (see below). For P1075 and (−)pinacidil, the inhibition curves reached 100% at saturation. In contrast, the inhibition curve of the benzopyran rilmakalim reached saturation at 92% (Figure 2). For aprikalim, saturation of the low-affinity phase could not be achieved (data not shown), but the curve extrapolated to 100% inhibition (Table 2).

In contrast to these 'typical' openers, which showed biphasic inhibition curves, minoxidil sulphate and nicorandil gave monophasic inhibition curves in the accessible concentration

range with amplitudes of 30±3 and 55±2%, respectively. With diazoxide, the inhibition curve at 300 μM was far from saturation and the curve was extrapolated to 100% (Figure 2, Table 2).

[^{3}H]GBC – opener inhibition assays were also conducted in the absence of MgATP (1 mM EDTA, no extra Mg^{2+} nor ATP). P1075 and (−)pinacidil inhibited [^{3}H]GBC binding completely, whereas the rilmakalim curve extrapolated to 79±1% (Figure 2, Table 2). With levcromakalim and aprikalim, saturation of inhibition could not be reached and curves were extrapolated assuming 100% inhibition at saturation. Surprisingly, inhibition by diazoxide was not affected by the withdrawal of MgATP, while minoxidil sulphate and nicorandil were unable to inhibit [^{3}H]GBC binding in the absence of MgATP.

Next, it was attempted to understand the biphasic nature of the [^{3}H]GBC inhibition curves for the 'typical' openers in the presence of MgATP. Inspection of Figure 2 and Table 2 shows that the K_i values of the high-affinity components were generally similar to the K_i values determined in the corresponding [^{3}H]P1075 competition assays (Table 1); the latter assays reflect the true affinity of the openers (Bray & Quast, 1992; Quast *et al*., 1993). Quantitatively, comparison of the respective pK_i values (excluding diazoxide as an obvious outlier) gave an excellent correlation ($r^2 = 0.98$) with the correlation line coinciding with the line of identity (Figure 3a). This showed that the high-affinity component of the [^{3}H]GBC – opener inhibition assays reflected opener affinity.

Figure 3b presents the comparison of the pK_i values from the low-affinity components of the inhibition curves in the presence of MgATP with those from the monophasic inhibition curves in the absence of MgATP. Minoxidil sulphate and nicorandil do not appear in the correlation since they were inactive in the absence and monophasic in the presence of MgATP. An excellent correlation ($r^2 = 0.92$) was obtained with slope unity, and diazoxide, which inhibited binding of [^{3}H]GBC in a manner independent of MgATP, was on the correlation line. Despite the slight offset from the line of identity by 0.25 log units (corresponding to a linear factor of 1.8), this suggested that these two components reflected the same process (see Discussion). It was also of interest to compare the potency of the openers in the [^{3}H]GBC binding assay in the absence of MgATP (Table 2) with the true affinity of the openers as determined in the [^{3}H]P1075 assay in the presence of MgATP (Table 2). Figure 3c shows that a good correlation ($r^2 = 0.98$) was obtained taking into account only the five 'typical' KCOs (i.e. excluding diazoxide). The correlation line had a slope of 1.2±0.1 and was shifted rightwards from the line of identity by ~2.3 log units. This indicated that, on average, the 'typical' openers were about 200× weaker in inhibiting [^{3}H]GBC binding in the absence of MgATP than in the [^{3}H]P1075 competition assay in the presence of MgATP.

Block of Kir6.2/SUR2B channels by 100 μM ATP in the absence of Mg^{2+}

To examine the effect of the KCOs on the Kir6.2/SUR2B channel in the absence of Mg^{2+}, openers were first applied to inside–out patches in the absence of ATP. Under these conditions, no further activation of the current but rather inhibition was often seen (not illustrated). The openers were therefore applied in the presence of ATP to keep the channels (partially) closed. A total of 1 mM ATP produced a sustained and complete channel block; however, addition of P1075 (100 μM) induced only small currents (8.4±2.5% of the control current in the absence of ATP; $n = 5$). In the presence of 100 μM ATP, P1075 (100 μM) gave larger responses (~32%, see below) and this ATP concentration was chosen for further experiments. The channel block induced by 100 μM ATP varied considerably. Figure 4a shows the distribution of the current remaining in the presence of 100 μM ATP (I_{ATP}) as constructed from 27 patches. About 50% of the patches were very sensitive to ATP ($I_{ATP} \leqslant 10\%$), whereas the other 50% were distributed over a wide range; the median of I_{ATP} was 11.5 (8,14)%. Figure 4b illustrates that the ATP sensitivity of a given patch varied little with time. Similar observations were made with the mutant channel, Kir6.2/SUR2B(Y1206S) (not shown).

Activation of Kir6.2/SUR2B channels by P1075

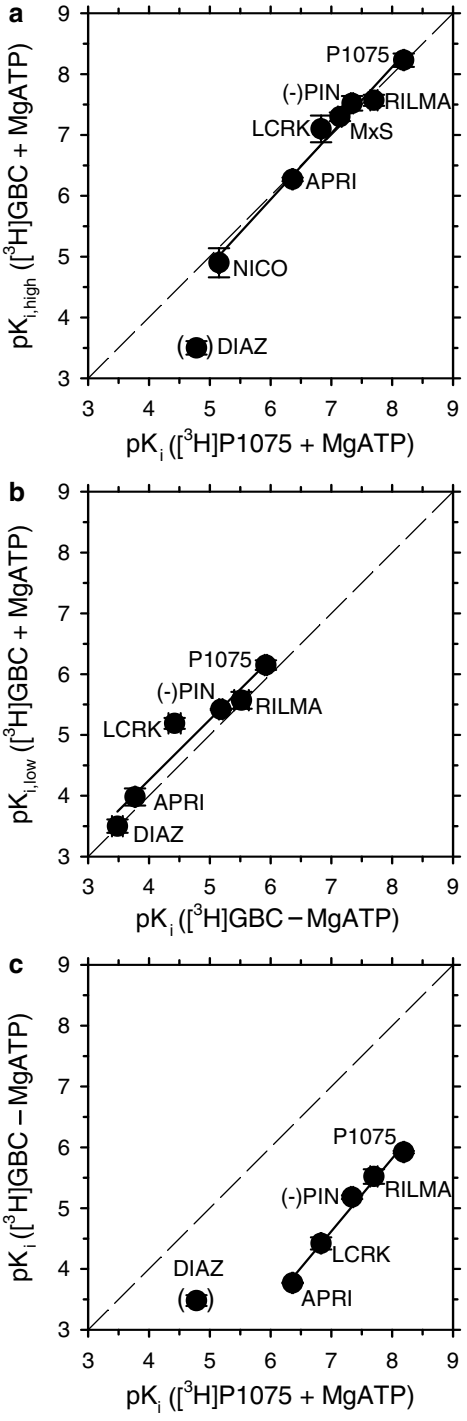
Experiments were first conducted in the absence of Mg^{2+}. Figure 5a shows a recording from a patch with good ATP

Table 2 Opener binding to SUR2B(Y1206S) as seen in [^{3}H]GBC competition assays in the presence and absence of MgATP (1 mM)

Opener	*+MgATP*		*–MgATP*		
	K_i	*A (%B_s)*	K_i *(μM)*	*A (%B_s)*	n_H
P1075[a]	5.9 (3.5, 9.8) nM	46±4	1.2 (1.1, 1.3)	100	1.1±0.1
	710 (490, 1023) nM	54±4			
(−)Pinacidil	30 (17, 50) nM	38±2	6.6 (5.7, 7.6)	100	1.1±0.1
	3.8 (3.6, 4.0) μM	62±2			
Rilmakalim	27 (18, 39) nM	42±4	3.0 (1.7, 5.2)	79±1	1.2±0.1
	2.7 (1.4, 5.1) μM	50±2			
LCRK	79 (29, 219) nM	38±8	38 (24, 60)	100[b]	1.4±0.2
	6.5 (4.3, 9.8) μM	57±5			
Aprikalim	540 (470, 620) nM	44±7	170 (160, 180)	100[b]	1.0±0.1
	105 (55, 200) μM	56±6			
Diazoxide	320 (190, 525) μM	100[b]	330 (220, 500)	100[b]	1.0[c]
Minoxidil sulphate	50 (36, 69) nM	30±3	At 300 μM:95% B_S		—
Nicorandil	13 (4, 36) μM	55±2	At 300 μM: 100% B_S		—

[a]Data for P1075 are from Hambrock *et al*. (2001). [b]Inhibition curves did not reach plateau at 300 μM of inhibitor and were extrapolated to 100% inhibition of B_S. [c]The Hill coefficient was fixed to 1.

sensitivity ($I_{ATP} = 3\%$). P1075 (10 μM), applied in the presence of ATP (100 μM), induced a current (I_{P1075}) which amounted to $\sim 70\%$ of that in the absence of ATP. I_{P1075} was reversibly inhibited by GBC (10 μM), suggesting that it was indeed a K_{ATP} channel current. After a washout phase of 10 min with repeated application of ATP (1 mM), P1075 (10 μM), applied 18 min after patch excision, was again effective; this time, I_{P1075} reached only $\sim 40\%$ of the current in the absence of ATP. This decrease in the efficacy of P1075 could reflect either specific desensitisation of the channel towards the opener upon repeated application or a more general kind of decrease in sensitivity towards KCOs occurring with time (run down).



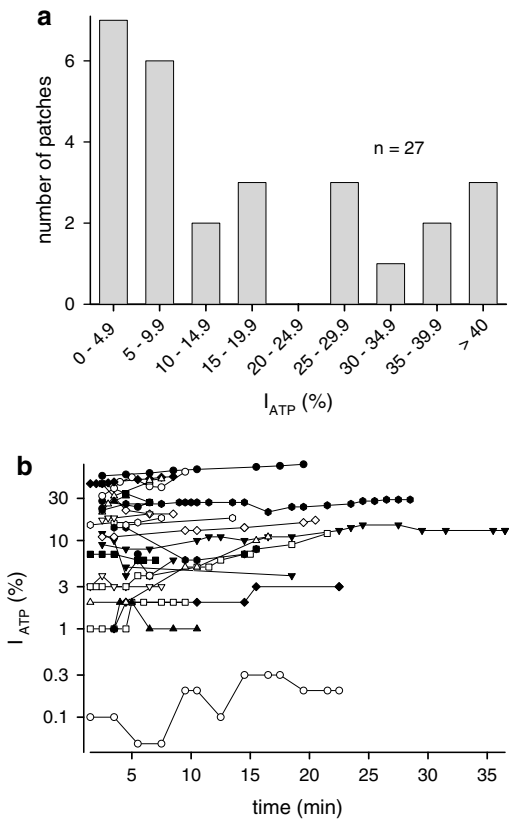


Figure 4 Block of the Kir6.2/SUR2B channel by ATP (100 μM) in the absence of ($[Mg^{2+}]_{free} \leqslant 10$ nM). (a) Distribution of K_{ATP} currents in the presence of 100 μM ATP (I_{ATP}) as determined from 27 patches. I_{ATP} was normalised with respect to the control current in the absence of ATP. (b) Dependence of I_{ATP} on the time of ATP application after patch excision for 27 patches.

In these experiments, it was noted that I_{P1075} was extremely variable, ranging between 2 and 80%. It was first examined whether the effect of P1075 depended on the time of drug application after patch excision (Figure 5b). Despite the enormous scatter in the data, the mean value of I_{P1075} averaged over the first 2 min where measurements were possible (i.e. 2 and 3 min after patch excision) was significantly higher than that in 15 – 20 min; this showed that there was a statistically significant decrease of I_{P1075} with time. When the data were

Figure 3 Pharmacological analysis of the pK_i values from the [^{3}H]glibenclamide ([^{3}H]GBC) – opener inhibition curves. (a) Comparison of the pK_i values from the high-affinity component ($pK_{i,high}$) in the presence of MgATP (1 mM) with the pK_i values from [^{3}H]P1075 – opener competition curves. The correlation line (slope = 1.1 ± 0.1; $r^2 = 0.98$) was not significantly different from the line of identity. (b) Comparison of the pK_i values from the low-affinity component ($pK_{i,low}$) in the presence of MgATP (1 mM) with pK_i from [^{3}H]GBC – opener inhibition curves in the absence of MgATP. The correlation line ($r^2 = 0.92$) had a slope of 1.0 ± 0.1 and was shifted leftwards from the line of identity by 0.25 log units, corresponding to a factor of 1.8 (0.9,3.4) on the linear scale. (c) Comparison of the pK_i values from the [^{3}H]GBC – opener inhibition assays in the absence of MgATP with the pK_i values from [^{3}H]P1075 – opener curves in the presence of MgATP (1 mM). The correlation line ($r^2 = 0.98$) had slope 1.2 ± 0.1 and was shifted leftwards from the line of identity by 2.3 log units (linear factor of 210 (126,350)).

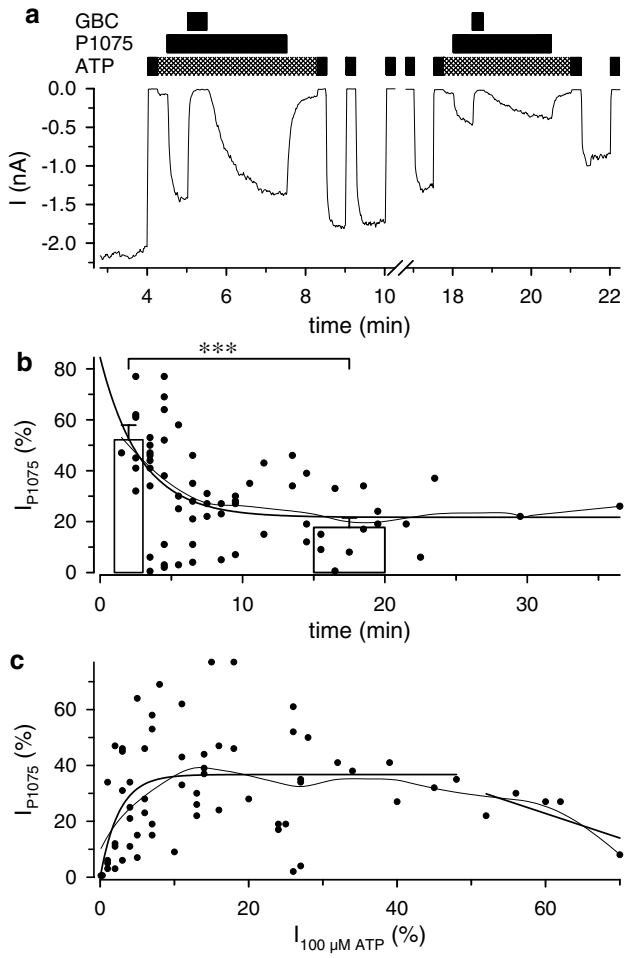


Figure 5 Activation of Kir6.2/SUR2B channels by P1075 (10 μM) in the absence of Mg^{2+}. (a) Original recording from a inside – out patch after excision into Mg^{2+}-free solution. ATP (1 mM (solid bar), 0.1 mM (crosshatched bar)), P1075 (10 μM) and GBC (10 μM) were applied as indicated by the bars. (b) Dependence of the P1075-induced current (I_{P1075}) on the time of P1075 application after patch excision. I_{P1075} was normalised with respect to the current in the absence of ATP; data represent 66 applications of P1075 to 27 patches. Values during the 1 – 3 and 15 – 20 min were normally distributed with the same variance and the respective means (open bars) were significantly different ($P=0.001$, t-test). (Thin curve) Data smoothing (SigmaPlot, bisquare weighting). (Thick curve) Fit of an exponential decay (Equation (3)) to the data; $k=0.35\pm0.18\,min^{-1}$, $a=63\pm31\%$ and $b=22\pm4\%$. The F value (9.2) suggested that the fit was meaningful ($P<0.001$). (c) Dependence of I_{P1075} on the current in the presence of 100 μM ATP (I_{ATP}). (Thin curve) Data smoothing. (Thick curve) Fit of an exponential growth to the data for $x<50\%$; $a=37\pm3\%$, $k=0.40\pm0.16$; the fit was highly significant ($F=16$; $P=0.0002$). Data for $x>50\%$ were analysed using a straight line (slope = – 0.9, ordinate intercept = 76%, $r^2=0.47$).

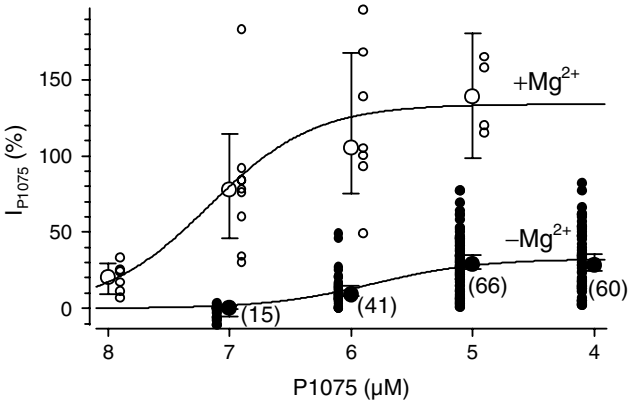


Figure 6 Dependence of I_{P1075} on P1075 concentration in the absence and presence of Mg^{2+}. P1075 was applied in the presence of 0.1/1 mM ATP in the absence/presence of Mg^{2+}, respectively (cf. Figures 5a and 7b). I_{P1075} was normalised with respect to the K_{ATP} current in the absence of ATP. Responses at a given P1075 concentration are shown as small symbols. They were not normally distributed and are also represented by the median (large circle) together with the 95% confidence interval; (n) indicates the number of experiments. Fits of the logistic function with the Hill coefficient 1 to all data points gave for the curves in the absence/presence of Mg^{2+} maximum effects of $32\pm2/134\pm13\%$ and midpoints (pEC_{50}) of $5.81\pm0.16/7.17\pm0.20$, respectively.

smoothed using the SigmaPlot programme, a curve resembling an exponential decay was obtained (Figure 5b). The fit of an exponential (Equation (3)) to the data showed that the efficacy of 10 μM P1075 decreased with a half-time of $\sim$2 min from an initial value of $85\pm31\%$ to a stable level of $24\pm4\%$. Similar data were obtained with 1 and 100 μM P1075; at 100 μM, the scatter was lower and the exponential fit gave parameters similar to those obtained in Figure 5b (not illustrated). This showed that the decrease in P1075 efficacy with time did not depend on the P1075 concentration applied; the decrease rather reflected a change in the state of the channel occurring after patch excision. It is concluded that the decrease in the sensitivity of the channel towards P1075 with time accounts for some of the variability apparent in Figure 5b.

It was also examined whether the variability of I_{P1075} was correlated with the ATP sensitivity of the channel. The idea behind this was that high concentrations of PIP_2 in the patch would decrease the sensitivity of the channel towards both ATP and the opener (Krauter *et al*., 2001). Figure 5c presents a graph of I_{P1075} vs the current remaining in the presence of 100 μM ATP (I_{ATP}). There is again a large scatter but one recognises a tendency for I_{P1075} to increase initially, to reach a plateau value and to decrease again at high values of I_{ATP} (thin line, obtained by data smoothing). Therefore, an exponential function was heuristically fitted to the data for $I_{ATP}<50\%$ and a straight line to the data for $I_{ATP}>50\%$.

The dependence of I_{P1075} on both the time of drug application and on the ATP sensitivity of channel eliminated only a small part of the total variability of the effect. For the analysis of the dependence of I_{P1075} on P1075, concentration data at each concentration were therefore pooled (Figure 6). The median of these values showed significant changes with P1075 concentration and the fit of the logistic equation with the Hill coefficient 1 taking all individual responses into account gave a maximum activation of $31\pm3\%$ of the K_{ATP} current in the absence of ATP with midpoint at $\sim$2 μM.

Since the binding experiments were carried out with mutant SUR2B some electrophysiological experiments were performed also with the mutant channel, Kir6.2/SUR2-B(Y1206S). Figure 7a presents a trace where P1075 (1 – 100 μM) was applied cumulatively to the same patch for four times. The experiment shows that the maximum response decreased with time and that the EC_{50} value of P1075 was

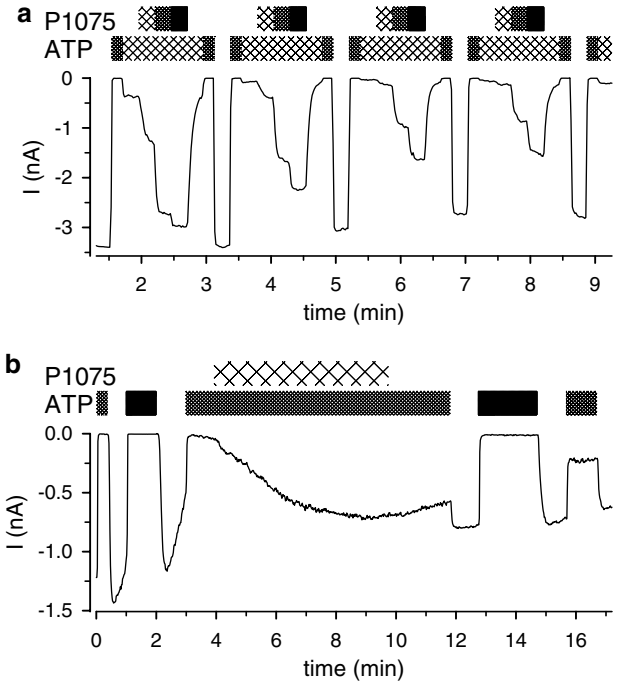


Figure 7 Original recordings illustrating the effects of P1075 on the mutant channel and in the presence of Mg^{2+}. (a) Mutant channel: repetitive cumulative stimulation of the Kir6.2/SUR2B(Y1206S) channel by P1075 in the presence of 100 μM ATP and the absence of Mg^{2+}. P1075 was 1, 10 and 100 μM (crosshatched coarse, fine and solid bars) and ATP was 0 (no bar), 0.1 and 1 mM (crosshatched coarse and fine bars, respectively). Note the decrease of channel responsiveness with time. (b) Effect of P1075 (0.1 μM) on the Kir6.2/SUR2B channel in the presence of $[Mg^{2+}]_{free} = 0.7$ mM; ATP was 0 (no bar), 1.0 (crosshatched) or 10 mM (solid bar). In the absence of ATP, rapid run down of the current occurred which was completely reversed upon exposure to MgATP. The P1075 (0.1 μM)-induced current was 60% of the control current immediately after ATP removal. Note the slow fading of the current upon washout of P1075.

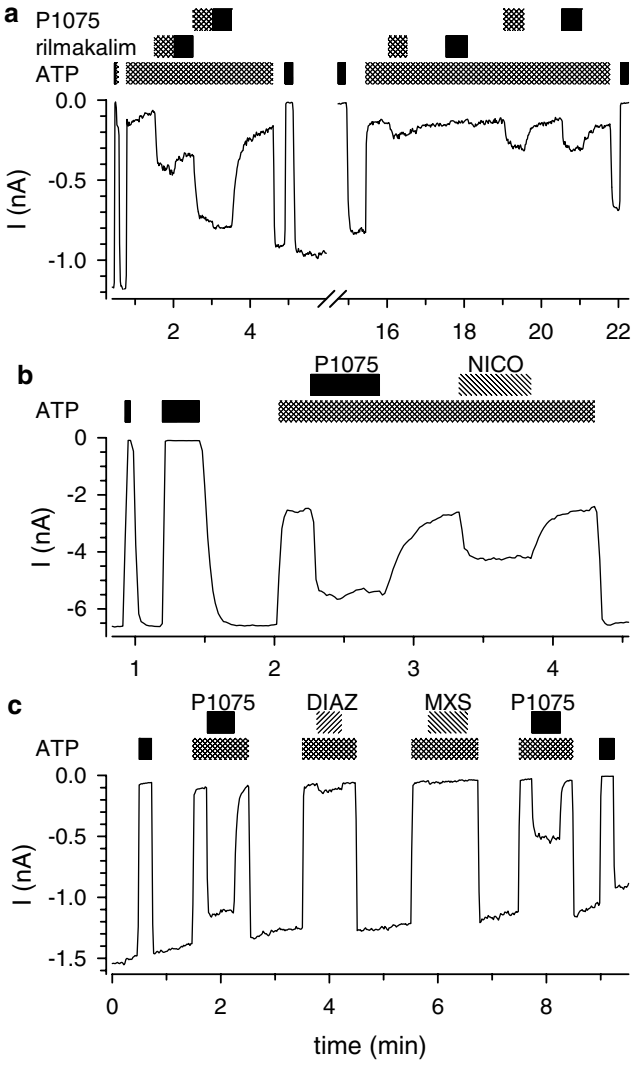


Figure 8 Effect of KCOs on Kir6.2/SUR2B channels. Original recordings from inside – out patches excised in Mg^{2+}-free buffer. ATP was 0.1 (crosshatched) and 1 mM (solid bar); P1075 (10 μM (crosshatched) or 100 μM (solid bar)) was applied for comparison. (a) Rilmakalim (10 μM (crosshatched bar) and 100 μM (solid bar)); (b) nicorandil (NICO, 1 mM); (c) diazoxide (DIAZ) and minoxidil sulphate (MXS) at 300 μM.

between 2 and 5 μM. These results were confirmed in seven experiments and they agreed with those obtained with the wild-type channel, as did the variability of the responses to P1075 from patch to patch.

Some experiments were also performed in the presence of Mg^{2+}, and the wild-type channel was used again. The block of the Kir6.2/SUR2B channel by MgATP decreases with time (Reimann *et al*., 2000) and the MgATP concentration was increased to 1 mM to keep the channel essentially closed. In order to avoid desensitisation of the channel to higher opener concentrations in the presence of MgATP (Shindo *et al*., 1998), patches were exposed to only one concentration of the opener. In the trace shown in Figure 7b, P1075 (100 nM) slowly induced a current which rose up to 60% of that in the absence of ATP. Upon switching to a pipe solution without P1075, the effect reversed only slowly. This showed that in the presence of Mg^{2+} (= MgATP), the washout of the opening effect was much slower that in its absence (see also Ashcroft & Gribble, 2000; Reimann *et al*., 2000). Even in the presence of 10 mM MgATP, residual channel activity was seen (Figure 7b). The concentration dependence of the P1075 effect in the presence of Mg^{2+} is shown in Figure 6 and gave an EC_{50} value of 67 (43, 110) nM with a maximum effect of 134 $\pm$ 13%.

Effect of other KCOs on Kir6.2/SUR2B in the absence of Mg^{2+}

Other KCOs were also examined in the presence of 100 μM ATP; representative traces are shown in Figure 8. As observed with P1075, responses were variable and they were divided into three groups (good, small and 0; Table 3). Rilmakalim was chosen as a representative of the benzopyran group of openers. Figure 8a shows that rilmakalim (10 μM) induced a larger current than 100 μM and that the efficacy of rilmakalim was subject to run down similar to P1075. Overall, 10 μM rilmakalim induced about half the current of 10 μM P1075 (Figure 8a, Table 3). At 1 μM, rilmakalim induced only small responses (< 5% of the current in the absence of ATP, $n = 4$). Nicorandil, at 100 μM, was barely active whereas at 1000 μM, the effect was comparable to that of 10 μM rilmakalim (Table 3). Diazoxide (300 μM) was less effective and gave no

Table 3 Response of Kir6.2/SUR2B channels to KCOs in the absence of Mg^{2+}

KCO (μM)	*Response*			*Total number of patches*
	Good I_{KCO} *(%)* *(n)*	*Small (<5%)* *(n)*	*0* *(n)*	*(n)*
P1075 (10)	31.4±2.3 (26[a])	1	—	27
Rilmakalim(10)[b]	18.4±2.8 (9[c])	2	—	11
Nicorandil (100)	6 (1)	1	1	3
Nicorandil(1000)	20.5±4.9 (8)	1	—	9
Diazoxide (300)	11.7±4.1 (3)	3	10	16
Minoxidil sulphate (300)	—	3	4	7

The current induced by the KCO in the presence of 100 μM ATP, I_{KCO}, was expressed in percent control current in the absence of ATP. For 'good' responses, I_{KCO} is given as the mean with the s.e.m. followed by the number of patches (*n*); for the small responses, only (*n*) is given. [a]Value calculated from 64 applications. [b]In five patches, rilmakalim as well as P1075 were applied and the effect of rilmakalim was always smaller (cf. Figure 8a). [c]Value calculated from 17 applications.

response in 63% of the experiments. Minoxidil sulphate (300 μM) showed only small or no responses. From the data in Table 3, the ability of the KCOs to activate the Kir6.2/SUR2B channel in the absence of Mg^{2+} decreases with the rank order P1075 > rilmakalim ~ nicorandil > diazoxide > minoxidil sulphate.

Discussion

Mutant vs wild-type SUR2B

The experiments comparing mutant and wild-type SUR2B showed that the mutation Y1206S has no major effect on the affinity of opener binding nor on the channel opening action of P1075. In addition, we had shown earlier that the mutation left the allosteric coupling between GBC and P1075 binding unchanged (Löffler-Walz *et al*., 2002). One can therefore safely assume that the [^{3}H]GBC – KCO inhibition experiments using mutant SUR2B truly reflect the interactions occurring with the wild-type.

Binding of 'typical' KCOs

In the presence of MgATP, the [^{3}H]GBC inhibition curves of the cyanoguanidines (P1075, (−)pinacidil), the benzopyrans (rilmakalim, levcromakalim) and of the thioformamide, aprikalim, were biphasic. In agreement with the model proposed earlier for P1075, it is assumed that SUR2B, expressed alone, forms tetramers with intersubunit coupling in the presence of MgATP (Löffler-Walz *et al*., 2002). The first component then reflects opener binding to the subunits not occupied by GBC. Indeed, the respective K_i values agreed with the K_i values from the [^{3}H]P1075 experiments and therefore gave the true affinity of the openers (Figure 3a). For P1075, we had shown that binding of this opener to the subunits not occupied by [^{3}H]GBC weakened the affinity of the radioligand by a negative allosteric interaction across subunits, increasing the K_D of [^{3}H]GBC binding by ~3 × and causing a reduction in binding of ~46%. The results in Table 2 show that this is true also for the other 'typical' openers.

The low-affinity component reflects opener binding to the subunit(s) occupied by [^{3}H]GBC. This occurs with lower affinity and leads to further displacement of [^{3}H]GBC from SUR by the negative allosteric interaction between the respective sites of the same subunit (Löffler-Walz *et al*., 2002). Indeed, the K_i values of the low-affinity component were close to those determined in the absence of MgATP (Figure 3b). With the exception of rilmakalim, inhibition reached 100%, that is, binding of the KCO to the subunit(s) occupied by [^{3}H]GBC induced complete dissociation of the radioligand. In the case of rilmakalim, ~10% [^{3}H]GBC remained bound at saturation, suggesting that the negative allosteric coupling between rilmakalim and GBC binding to the same subunit is less tight than in case of the other 'typical' KCOs.

In the absence of MgATP, the [^{3}H]GBC inhibition curves of the 'typical' KCOs were monophasic. Within the framework of the tetramer model, it is assumed that in the absence of MgATP, no intersubunit interactions occur (Löffler-Walz *et al*., 2002). Hence, binding of KCOs to the subunits not occupied by [^{3}H]GBC is mute. Binding of the KCOs to the subunits occupied by [^{3}H]GBC, however, gives information on the negative allosteric interaction between opener and GBC binding at this subunit. Correlation analysis showed that the five 'typical' openers inhibited [^{3}H]GBC binding with K_i values approximately 210 times higher than their K_D value determined in the presence of MgATP (Figure 3c). At first sight this seems to indicate that MgATP increases the affinity of SUR for the 'typical' KCOs 210-fold. However, one has to realise that the signal does not reflect opener binding to an empty SUR subunit but to one occupied by GBC. The presence of GBC at the same subunit renders opener binding difficult and *vice versa* (Hambrock *et al*., 2001). Hence, these experiments are likely to underestimate the true affinity of these KCOs for the opener site in the absence of MgATP. This idea is supported by the fact that the K_i values in the absence of MgATP agreed (within a factor of 1.8) with those from the low-affinity component of the inhibition curves in the presence of MgATP (Figure 3b). This showed that at the subunit(s) occupied by GBC, the presence of GBC was more important for opener binding than the presence of MgATP. In addition, MgATP has been shown to slow down the reversal of the pinacidil effect at the Kir6.2/SUR2B channel upon washout by ~14 times (at 22°C, Reimann *et al*., 2000), which is less than the difference in K_i of 210 obtained here from the [^{3}H]GBC inhibition curves (at 37°C).

Binding of 'atypical' KCOs

In the presence of MgATP, the [^{3}H]GBC inhibition curves of diazoxide, minoxidil sulphate and nicorandil were monophasic in the accessible concentration range; hence, these openers were called here 'atypical' KCOs. For minoxidil sulphate and nicorandil, the K_i values agreed with those from the [^{3}H]P1075 experiments (Figure 3a). This showed that these inhibition curves corresponded to the high-affinity component of the biphasic curves of the 'typical' KCOs and that the low-affinity component was missing. In agreement with this analysis, these

two openers did not inhibit [^{3}H]GBC binding in the absence of MgATP. It is concluded that either minoxidil sulphate and nicorandil did not bind to the subunit occupied by [^{3}H]GBC or that their binding did not induce dissociation of [^{3}H]GBC.

Diazoxide, in contrast, inhibited [^{3}H]GBC binding in a manner independent of MgATP. Correlation analysis (Figure 3b) indicated that the inhibition curve corresponded to the low-affinity component of the biphasic curves of the 'typical' openers and that the high-affinity component was lacking. Hence, this curve reflected the interaction of diazoxide with the subunit(s) occupied by GBC. The lack of the high-affinity component in the presence of Mg^{2+} sets diazoxide apart from the other KCOs tested here and suggests that this opener has a special mode of binding to SUR2B. This idea is supported by electrophysiological studies using chimeras between SUR2A and SUR1 and special point mutations at both subtypes, which have provided direct evidence that the interaction of diazoxide with SUR differs from that of benzopyrans and cyanoguanidines (D'hahan *et al*., 1999a; Babenko *et al*., 2000; Moreau *et al*., 2000).

Channel opening

A major point of this study is that P1075 and other KCOs open the Kir6.2/SUR2B channel after exposure to a Mg^{2+}-free solution ($[Mg^{2+}]_{free} < 10$ nM, see Methods) for up to 30 min. It is therefore highly unlikely that, in our experiments, the KCOs acted by stabilising the posthydrolytic state of SUR with MgADP bound at NBD2 (Zingman *et al*., 2001). In contrast, the results presented here and by others (see Introduction) strongly suggest that KCOs can activate the channel by a pathway independent from ATP hydrolysis. However, one cannot exclude the possibility that ATP^{4-}, by binding to NBD1 (Matsuo *et al*., 2000), may somehow 'condition' the channel for the action of the openers.

Comparing the channel activating curves in Figure 6, one notes that in the presence of 1 mM MgATP, P1075 opens the channel with $\sim$4 times higher efficacy and $\sim$30 times higher potency than in the absence of MgATP (0.1 mM ATP). The potency of P1075 in the presence of MgATP agrees with that reported by Schwanstecher *et al*. (1998) (45 nM). Interestingly, the opener concentrations used here in the absence of MgATP were similar or even identical to those used to delineate the ATPase-dependent pathway of channel opening (Bienengraeber *et al*., 2000; Zingman *et al*., 2001), and high KCO concentrations are often used in the presence of MgATP. One may ask to which degree the ATPase-independent pathway then contributes to the overall channel activating effect. If this pathway was still operative, the concentration – response curve might be biphasic with components centred around 70 nM and 2 μM. Obviously, the scatter of the data in Figure 6 (each patch was exposed only to one concentration of P1075 to avoid desensitisation) does not allow a conclusion to be drawn and the Hill coefficient carries a large error ($n_H = 0.9 \pm 0.4$). In addition, one has to consider that the experiments were performed at 1 mM ATP, a concentration at which the Mg^{2+}-independent effect of the openers is much smaller than at 0.1 mM ATP (see Results). The latter observation suggests that under physiological conditions, the Mg^{2+}-independent pathway does not play a significant role, even at high opener concentrations.

Binding and effect

In the presence of Mg^{2+} (i.e. MgATP), P1075 bound to SUR2B with $K_D = 4.4$ nM and activated the Kir6.2/SUR2B channel with $EC_{50} = 67$ nM, that is, the channel activation curve was shifted to the right of the binding curve by a factor of $\sim$15. A rightward shift of $\sim$4 was found by Schwanstecher *et al*. (1998) working under similar conditions and, for pinacidil, one calculates a rightward shift of 14 (K_i (binding) $\sim 0.1\,\mu$M (this study); $EC_{50} = 1.4\,\mu$M in whole cells; Shindo *et al*., 1998). The rightward direction of these shifts is remarkable. Gross *et al*. (1999) have demonstrated that occupation of one opener site out of four is sufficient for channel opening; however, assuming the channel is endowed with four equal and independent opener binding sites, one calculates for this stoichiometry that the channel opening curve lies at the left of the binding curve by a factor of $(2^{1/4} - 1)^{-1} = 5.3$ (Gross *et al*., 1999; Russ *et al*., 1999). One explanation for this apparent contradiction is that in the presence of MgATP, the openers appear to activate the channel by modulating the ATPase activity of SUR (Bienengraeber *et al*., 2000). Hence, there is a constant influx of energy into the system, which then operates at steady state and considerable deviations from the behaviour at equilibrium occur (Boeynaems & Dumont, 1980; Löffler-Walz *et al*., 2002).

In contrast, in the absence of Mg^{2+} and for the two KCOs for which data are available (P1075 and rilmakalim), the concentration range of binding agreed well with that of channel activation (P1075: $K_i = 1\,\mu$M, EC_{50} $\sim 2\,\mu$M; rilmakalim: $K_i = 3\,\mu$M; optimum effect concentration 10 μM). Although the comparison of these values is hampered by the different assay conditions (binding studies: mutant SUR2B without coexpression, use of GBC as the radioligand, absence of ATP, assay temperature 37°C) and by the variability of the opener effect in the electrophysiological experiments, this agreement is surprising. For two reasons one might expect to find the channel activation curve at the left of the binding curve: first, in the absence of Mg^{2+}, no hydrolysis of ATP should occur and the system operates at equilibrium. Hence, the stoichiometry argument explained above – if it holds in the absence of MgATP – predicts a leftward shift of the channel activation curve by a factor of 5.3. Second, it was argued above that the K_i values determined in [^{3}H]GBC binding experiments underestimate the true affinity of the openers (see above). Potential explanations for the unexpected agreement are that in the absence of MgATP, more than one opener site must be occupied for channel opening or that the mechanism of channel opening is complex, even in the absence of ATPase activity.

Other openers

The ability of the KCOs to open the Kir6.2/SUR2B channel in the absence of MgATP ranks them in the order P1075 > rilmakalim $\sim$ nicorandil > diazoxide > minoxidil sulphate. Regarding rilmakalim, we recall that the negative allosteric coupling between rilmakalim and GBC binding was weaker than that observed for the other 'typical' openers (see above). Interestingly, rilmakalim was also less efficient than P1075 in opening the Kir6.2/SUR2B channel in the absence of MgATP.

Regarding the 'atypical' KCOs, it is worth noting that nicorandil was mute in the [^{3}H]GBC binding assay but quite

effective in opening the channel. A similar discrepancy was found for minoxidil sulphate; however, the effect of this opener (at 300 μM) was marginal. One has to conclude that these compounds bind to the channel without affecting [^{3}H]GBC binding (see above). The efficacy of nicorandil observed here for Kir6.2/SUR2B in the absence of Mg^{2+} may be limited to this channel subtype since in the presence of MgATP, nicorandil is more effective on SUR2B than on SUR2A (Shindo *et al*., 1998; Reimann *et al*., 2001). Interestingly, diazoxide, which activates the Kir6.2/SUR2A channel only in the presence of MgATP and of supraphysiological concentrations of MgADP (D'hahan *et al*., 1999b), was able to activate the Kir6.2/SUR2B channel in the absence of MgATP.

Variability in channel responsiveness to KCOs

A striking feature in the electrophysiological experiments in the absence of Mg^{2+} was the variability of the KCO effect from patch to patch. Two mechanisms were identified that contributed to a small but significant degree to this variability: channel responsiveness decreased with the time after patch excision and depended on the ATP sensitivity of the channel. The decrease in channel responsiveness with time was independent of the opener (P1075 or rilmakalim) or the concentration of opener applied, occurred with a half-time of $\sim$2 min and levelled off at $\sim$20% of the current in the absence of ATP. A similar decrease has been reported for pinacidil activating the Kir6.2/SUR2A channel in nucleotide-free but Mg^{2+}-containing medium (Gribble *et al*., 2000). In both cases (absence of Mg^{2+} or nucleotides), phosphorylation was prevented. Hence, the decrease in channel responsiveness could reflect, among other possibilities, the decay of important modulatory membrane phospholipids (PIP_2 or PIP_3), the dephosphorylation of a channel subunit or the release of an ATP hydrolysis product such as MgADP from SUR.

Turning to the relation between channel responsiveness towards P1075 and channel sensitivity towards ATP, one first states a difference in the time-dependence of the phenomena: the sensitivity for the opener decreased with time (Figure 5b), whereas ATP- sensitivity did not (Figure 4b). Membrane phospholipids like PIP_2 are prime determinants of the sensitivity of K_{ATP} channels towards ATP (Hilgemann & Ball, 1996; Baukrowitz *et al*., 1998; Shyng & Nichols, 1998), and KCOs (Krauter *et al*., 2001), and it has been proposed that the large variability in ATP-sensitivity is caused by the differences in the PIP_2 concentration present in the patch (Baukrowitz *et al*., 1998; Shyng & Nichols, 1998; Krauter *et al*., 2001). If PIP_2 is indeed the dominant modulator of ATP sensitivity, the lack of time dependence (Figure 4b) suggests that the PIP_2 concentration in the patch did not decrease significantly with time under the Mg^{2+}-free conditions employed here.

Despite the difference in time dependence there was a significant correlation of opener–with ATP-sensitivity. When the latter was assessed inversely by the current remaining in the presence of 100 μM ATP (I_{ATP}), P1075 responsiveness first increased with I_{ATP} to reach a plateau and then decreased again (Figure 5c). One may speculate that at low I_{ATP} (high ATP sensitivity), channel inhibition by ATP was too strong to be overcome by the opener. At high I_{ATP}, saturation of channel activation may be reached (as we have not observed that the opener increased channel activity beyond the level seen in the absence of ATP), leading to a linear decrease of the P1075 effect with I_{ATP}. Alternatively, and in consideration that low levels of PIP_2 are required to maintain channel activity and high levels inhibit the effect of P1075 (Krauter *et al*., 2001), the two limbs of the curve may reflect increasing PIP_2 levels in the low and the high concentration range of PIP_2. Whatever the case, one has to concede that neither the dependence of the opener effect on time nor on ATP sensitivity can satisfactorily account for the great variability of this effect which seems to critically depend on a parameter poorly controlled in our preparation.

In conclusion, we have shown that in the absence of Mg^{2+}, openers bind to (mutant) SUR2B and activate the Kir6.2/SUR2B channel by a mechanism independent of ATP hydrolysis. The concentrations required are similar to those which modulate the ATPase activity of SUR. The openers differ in their ability to activate the MgATP-independent pathway, and the mode of action of diazoxide differs from that of the 'typical' openers.

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