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Incomplete dissociation of glibenclamide from wild-type and mutant pancreatic K_{ATP} channels limits their recovery from inhibition

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Background and purpose: The antidiabetic sulphonylurea, glibenclamide, acts by inhibiting the pancreatic ATP-sensitive K^+ (K_{ATP}) channel, a tetradimeric complex of $K_{IR}6.2$ and sulphonylurea receptor 1 $(K_{IR}6.2/SUR1)_4$. At room temperature, recovery of channel activity following washout of glibenclamide is very slow and cannot be measured. This study investigates the relation between the recovery of channel activity from glibenclamide inhibition and the dissociation rate of [^{3}H]-glibenclamide from the channel at 37°C.
Experimental approach: $K_{IR}6.2$, $K_{IR}6.2\Delta N5$ or $K_{IR}6.2\Delta N10$ (the latter lacking amino-terminal residues 2–5 or 2–10 respectively) were coexpressed with SUR1 in HEK cells. Dissociation of [^{3}H]-glibenclamide from the channel and recovery of channel activity from glibenclamide inhibition were determined at 37°C.
Key results: The dissociation kinetics of [^{3}H]-glibenclamide from the wild-type channel followed an exponential decay with a dissociation half-time, $t_{1/2}(D) = 14$ min; however, only limited and slow recovery of channel activity was observed. $t_{1/2}(D)$ for $K_{IR}6.2\Delta N5$/SUR1 channels was 5.3 min and recovery of channel activity exhibited a sluggish sigmoidal time course with a half-time, $t_{1/2}(R) = 12$ min. $t_{1/2}(D)$ for the ΔN10 channel was 2.3 min; recovery kinetics were again sigmoidal with $t_{1/2}(R)$ ~4 min.
Conclusions and implications: The dissociation of glibenclamide from the truncated channels is the rate-limiting step of channel recovery. The sigmoidal recovery kinetics are in quantitative agreement with a model where glibenclamide must dissociate from all four (or at least three) sites before the channel reopens. It is argued that these conclusions hold also for the wild-type (pancreatic) K_{ATP} channel.
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Abbreviations: K_{ATP} channel, ATP-sensitive K^+ channel; SUR, sulphonylurea receptor

Introduction

ATP-sensitive K^+ channels (K_{ATP} channels) are a family of K^+ channels that are closed by intracellular MgATP and opened by MgADP and other nucleoside diphospho-Mg^{2+}-salts (Seino and Miki, 2003). By this unique property they link membrane potential to cellular metabolism. They are best known for their role in the pancreatic β-cell where they link the secretion of insulin to the plasma glucose level. The antidiabetic sulphonylureas were developed before K_{ATP} channels were discovered (Loubatières, 1957) and have since been found to act by inducing closure of the channel, thereby depolarizing the β-cell and initiating exocytotic release of insulin (Sturgess *et al.*, 1985; Gribble and Reimann, 2003; Bryan *et al.*, 2005).

K_{ATP} channels are composed of pore-forming subunits $K_{IR}6.x$ and sulphonylurea receptors (SURx); for the pancreatic channel, which is composed of $K_{IR}6.2$ and SUR1, a tetradimeric stoichiometry of the subunits, $(K_{IR}6.2/SUR1)_4$, has been shown (Clement IV *et al.*, 1997; Shyng and Nichols, 1997; Mikhailov *et al.*, 2005). $K_{IR}6.x$ are members of the inwardly rectifying K^+ channel superfamily and MgATP exerts its inhibitory function by binding to $K_{IR}6.x$ (Tucker *et al.*, 1997). SURs are members of the ATP-binding cassette protein superfamily (Aguilar-Bryan *et al.*, 1995); MgATP binding to and hydrolysis by SUR eventually opens the channel by outweighing channel inhibition by ATP via $K_{IR}6.x$ (Gribble *et al.*, 1998; Ueda *et al.*, 1999; Babenko, 2005; 2008; Nichols, 2006). The antidiabetic sulphonylureas bind to SUR (with a minor contribution of $K_{IR}6.2$ to the binding site; Vila-Carriles *et al.*, 2007; Winkler *et al.*, 2007) to close the channel.

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Sulphonylureas have been found to inhibit the pancreatic channel by two mechanisms. First, sulphonylureas stabilize the long-lived interburst closed state; second, they disrupt the activating input from SUR1 on K_{IR} (Gribble *et al.*, 1997; Babenko *et al.*, 1999b; Koster *et al.*, 1999a; Reimann *et al.*, 1999).

In this study, we sought to investigate the relation between the dissociation kinetics of a sulphonylurea from the $K_{IR}6.2$/SUR1 complex and the recovery of channel activity. The dissociation kinetics were measured using the radioligand binding technique. The only radiolabelled sulphonylurea available to date is [^{3}H]-glibenclamide that has a high affinity for the pancreatic channel (<1 nmol·L^{-1}; Schwanstecher *et al.*, 1991; Hansen *et al.*, 2005; Stephan *et al.*, 2006). At room temperature, at which electrophysiological experiments are generally performed, the dissociation half-time of glibenclamide from the channel is >1 h (Schwanstecher *et al.*, 1992) and channel inhibition is 'irreversible', that is, recovery of channel activity is not observed on the time scale of an electrophysiological experiment (Ashfield *et al.*, 1999). Hoping for faster dissociation and recovery at 37°C, experiments at this more physiological temperature gave exponential dissociation kinetics with a half-time of ~14 min; however, spontaneous run-down of channel activity was greatly increased and little (if any) recovery of channel activity was observed during 15 min of washout. Using N-terminally truncated $K_{IR}6.2$ with deletion of amino acids 2–5 ('$K_{IR}6.2\Delta N5$') or 2–10 ('$K_{IR}6.2\Delta N10$'), we observed faster dissociation kinetics and a slow sigmoidal recovery of channel activity. The recovery kinetics can be quantitative explained if one assumes that dissociation of glibenclamide from the channel is the rate-limiting step of the recovery process and that only channels completely free of glibenclamide or with at least three unoccupied SURs can reopen.

Methods

Molecular biology, cell culture, transfection and membrane preparation

Mouse $K_{IR}6.2$ (GenBank D50581) and rat SUR1 (GenBank X97279) were in the pcDNA 3.1 vector [receptor and channel nomenclature conforms to the BJP's Guide to Receptors and Channels, (Alexander *et al.*, 2008)]. The deletion of amino acids 2–5 or 2–10 from the N-terminus of $K_{IR}6.2$ ($K_{IR}6.2\Delta N5$ and $K_{IR}6.2\Delta 10$) was introduced into the human $K_{IR}6.2$ cDNA (OMIM 600937) in the pECE vector as described (Babenko *et al.*, 1999a). HEK 293 cells were cultured in minimum essential medium containing glutamine and supplemented with 10% foetal bovine serum and 20 μg·mL^{-1} gentamycin as described (Hambrock *et al.*, 2001). Cells were transfected with wild-type $K_{IR}6.2$ and SUR1 (both in pcDNA3.1) at a molar ratio of 1:1 and with $K_{IR}6.2\Delta N5$ or ΔN10 (pECE) and SUR1 (pcDNA3.1) at a molar ratio of 100:1, using Lipofectamine 2000 and Opti-MEM (Invitrogen) according to the manufacturer's instructions. Crude membranes were prepared as described by Hambrock *et al.* (1998) and frozen at –80°C. Protein concentration was determined according to Lowry *et al.* (1951) using bovine serum albumin as the standard.

Kinetics of [^{3}H]-glibenclamide binding

Membranes were thawed and homogenized with a Polytron homogenizer for 2 × 5 s at 10 000 r.p.m. and 4°C. To measure the association kinetics, membranes (final protein concentration 50–100 μg·mL^{-1}) were added to the incubation buffer containing (in mmol·L^{-1}): NaCl, 139; KCl, 5; HEPES, 5; $MgCl_2$, 2; Na_2ATP, 1; Na_2GDP, 0.3 (free [Mg^{2+}] ~0.8 mmol·L^{-1}) and preincubated for 5 min at 37°C and pH 7.4; association was started by adding [^{3}H]-glibenclamide (0.9–3 nmol·L^{-1}). Dissociation of [^{3}H]-glibenclamide from the channel was initiated by addition of unlabelled glibenclamide (10–100 nmol·L^{-1}) to the complex after equilibrium was reached or by 10-fold dilution of the complex. Aliquots (300 μL) were withdrawn at different times for separation of bound and free ligand by dilution into 8 mL of quench solution (50 mmol·L^{-1} TRIS, 154 mmol·L^{-1} NaCl, pH 7.4, 0°C) and rapid filtration under vacuum over Whatman GF/B filters. Filters were washed twice with 8 mL of quench solution at 0°C and counted for ^{3}H in the presence of 6 mL of scintillant (Ultima Gold; Packard). Nonspecific binding was determined in the presence of 100 nmol·L^{-1} unlabelled glibenclamide and was between 10–20% of total binding.

Patch-clamp experiments

Experiments in the whole-cell configuration of the patch-clamp technique were performed at 37°C as described by Russ *et al.* (1999). The bath was filled with (in mmol·L^{-1}): NaCl, 142; KCl, 2.8; $MgCl_2$, 1; $CaCl_2$, 1; D(+)-glucose, 11; HEPES, 10; pH 7.4. Patch pipettes were filled with (in mmol·L^{-1}) K-glutamate, 132; NaCl, 10; HEPES, 10; EGTA [ethylene glycol-bis-(2-aminoethylether)-N,N,N′,N′-tetraacetic acid], 1; MgATP, 1 and MgGDP, 0.3 at pH 7.2; $[Mg^{2+}]_{free}$ was ~0.85 mmol·L^{-1}. After filling, pipettes had a resistance of 3 to 5 MΩ. Cells were clamped at –60 mV. To examine the quality of the patch, every 12 s, seven square pulses ranging from –110 to 10 mV and lasting 0.5 s each were applied. Series resistance was compensated by 70%. After breaking into the cell and reaching a stable current, glibenclamide (30 nmol·L^{-1}) was applied to the bath for 5 min, washed out and recovery of the current was monitored.

Experiments in the inside-out patch-clamp configuration were performed using a home-made heating device that allowed rapid heating of the small solution volume around the tip of the patch pipette and of the application pipe from 22°C to 37°C (Russ and Quast, 2008). Bath and pipette were filled with a high K^+-Ringer solution containing (in mmol·L^{-1}) KCl, 142; NaCl, 2.8; $MgCl_2$, 1; $CaCl_2$, 1; D(+)-glucose, 11; HEPES, 10; titrated to pH 7.4 with NaOH. After filling with buffer, pipettes had a resistance of 1.0–1.5 MΩ. After patch excision, the pipette was moved in front of a pipe filled with a high K^+-EGTA-buffered solution containing (in mmol·L^{-1}) KCl, 143; $CaCl_2$, 1; D(+)-glucose, 11; MgATP 0 or 1, MgUDP, 0.3; EGTA, 5; HEPES, 10; $[Mg^{2+}]_{free}$ was ~0.85 mmol·L^{-1}. The buffer was titrated to pH 7.2 with NaOH at 22°C. Upon heating to 37°C pH shifted to 7.0, a shift that has little impact on channel activity (Baukrowitz *et al.*, 1999). Patches were clamped at –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, modelling and statistics

[^{3}H]-glibenclamide association kinetics were performed with large excess of ligand over receptor and fitted to an ascending exponential function

$$c(t) = c_\infty[1 - \exp(-k_{app}t)] + \text{NSB} \quad (1)$$

with $c(t)$ denoting the complex concentration as function of time (t), c_∞ the complex concentration at equilibrium, k_{app} the apparent rate constant of association and NSB nonspecific binding (determined in the presence of 100 nmol·L^{-1} glibenclamide). In case of a second order reaction (receptor + ligand ↔ complex) and a large excess of ligand, k_{app} is related to the rate constants of association (k_+) and dissociation (k_-) by

$$k_{app} = k_+L + k_- = k_-(1 + LK_D^{-1}) \quad (2)$$

(Tallarida, 1995), with L denoting ligand ([^{3}H]-glibenclamide) concentration and $K_D = k_-\ k_+^{-1}$ the equilibrium dissociation constant of binding.

The kinetics of complex dissociation were fitted to an exponential of the form

$$c(t) = c_\infty \exp(-k_-t) + \text{NSB} \quad (3a)$$

The half-time of dissociation [$t_{1/2}$(D)] is then related to the dissociation rate constant k_- by

$$t_{1/2}(\text{D}) = \ln 2k_-^{-1} = 0.69k_-^{-1} \quad (3b)$$

In the equilibrium and the kinetic experiments, no systematic deviation of the data from these equations was observed, suggesting that the four glibenclamide binding sites in the tetradimeric channel complex are identical and independent (Dörschner *et al.*, 1999).

In modelling the recovery of channel activity upon washout of glibenclamide, we assume that at time 0 when washout begins all four sites are occupied by glibenclamide. Let p denote the probability that at time t a site is free from glibenclamide, we have

$$p = 1 - \exp(-k_-t) \quad (4a)$$

(cf. Eqn 3a). Let α_i denote the open probability of a complex with i free subunits ($0 \le i \le 4$) normalized with respect to that of the free channel so that $0 \le \alpha_i \le 1$. Recovery of channel activity (y) then follows the time course

$$y = y_{max}\sum_{i=0}^{4}\alpha_i\binom{4}{i}p^i(1-p)^{4-i} \quad (4b)$$

with y_{max} denoting channel activity after complete recovery and $\alpha_4 = 1$.

Letting α_i float freely did not give consistent results. Therefore, discrete values were assigned to α_i ($\alpha_i = 0,\ 1,\ i/4$). A simple case is the 'graded' model, in which each subunit that is freed from glibenclamide increases the open probability of the tetradimeric channel by 1/4 of the value in the absence of glibenclamide ($\alpha_i = i/4$). Recovery of channel activity is then described by Eqn 4a. Another simple case is the four-site model in which only channels with all four SUR subunits freed from glibenclamide regain activity (i.e. $\alpha_i = 0$ for $i = 0$–3; four-site model; Dörschner *et al.*, 1999). Recovery of channel activity (y) then follows the equation

$$y = y_{max}[1 - \exp(-k_-t)]^4 \quad (4c)$$

and the half-time of recovery [$t_{1/2}$(R)] is calculated to be

$$t_{1/2}(\text{R}) = -\ln(1 - 2^{-1/4})k_-^{-1} = 1.84k_-^{-1} \quad (4d)$$

Comparison with Eqn 3b shows that

$$t_{1/2}(\text{R}) = 2.7t_{1/2}(\text{D}) \quad (5)$$

In general, if glibenclamide must have dissociated from *at least i* SUR1 subunits for the channel to open with the open probability in the absence of glibenclamide (regardless of the state of the other 4 – i subunits), we have

$$y = y_{max}[1 - \exp(-k_-t)]^i \quad (1 \le i \le 4) \quad (6)$$

If dissociation of one glibenclamide is sufficient for the channel to open again (one-site model), this reduces to Eqn 4a and gives recovery kinetics identical to the 'graded' model.

Equations were fitted to the data according to the method of least squares using the program SigmaPlot 9.0 (SPSS Science, Chicago, IL). Significance of differences between two normally distributed parameters with equal variance was assessed using the two-tailed unpaired Student's t-test using the program SigmaStat 3.1 (SPSS Science, Chicago, IL).

Materials

[^{3}H]-glibenclamide (specific activity 1.85 TBq·mmol^{-1}) was purchased from Perkin-Elmer Life Sciences (Bad Homburg, Germany). The reagents and media used for cell culture and transfection were from Invitrogen (Karlsruhe, Germany). Glibenclamide was purchased from Sigma (Deisenhofen, Germany). Glibenclamide was dissolved in dimethyl sulphoxide/ethanol (50/50, v/v) and further diluted with the same solvent or with incubation buffer (final solvent concentration in the assays <1%).

Results

Experiments with the K_{IR}6.2/SUR1 (wild-type) channel

Figure 1A presents the kinetics of association of [^{3}H]-glibenclamide to and dissociation from the channel at 37°C. Dissociation was initiated by addition of unlabelled glibenclamide (10–100 nmol·L^{-1}) and followed an exponential function with a rate constant of 0.049 ± 0.001 min^{-1} (n = 4) corresponding to a half-time $t_{1/2}$(D) = 14 min. Figure 1B shows an electrophysiological experiment carried out at 37°C. Upon dialysis of the cell with an activating nucleotide solution (1 mmol·L^{-1} MgATP + 0.3 mmol·L^{-1} MgGDP, 0.7 mmol·L^{-1} $[Mg^{2+}]_{free}$), K_{ATP} channels opened partially and subsequently ran down. The run-down process is presumably due to hydrolysis of phosphoinositides and possibly de-phosphorylation of the channel

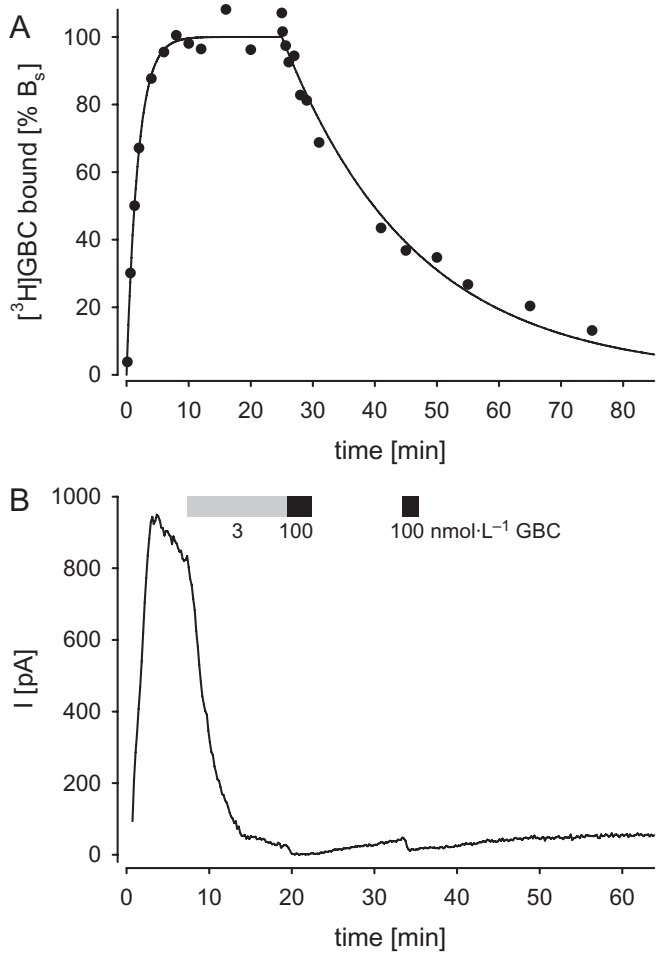

Figure 1 Experiments with $K_{IR}6.2$/SUR1. a. Association and dissociation of kinetics of [^{3}H]-glibenclamide ([^{3}H]-GBC) in the presence of 1 mmol·L^{-1} MgATP at 37°C. Association was measured in the presence of [^{3}H]-glibenclamide = 0.73 nmol·L^{-1}. The kinetics were described by an exponential function (Eqn 1 in *Methods*) and gave an apparent rate constant of association (k_{app}) of 0.54 ± 0.03 min^{-1}. Dissociation was initiated by addition of 100 nmol·L^{-1} glibenclamide and the fit of the data to Eqn 3a gave the dissociation rate constant, k_{-}(D), a value of 0.047 ± 0.003 min^{-1} corresponding to a half-time of 15 min. b. Channel inhibition by glibenclamide and washout measured in the whole-cell configuration at −30 mV and 37°C. Dialysis of the cell with 1 mmol·L^{-1} MgATP and 0.3 mmol·L^{-1} MgGDP elicited a current that showed some run-down. Glibenclamide (3 and 100 nmol·L^{-1}) was applied to the bath as indicated by the grey (3 nmol·L^{-1}) and black bars (100 nmol·L^{-1}). Note the very small recovery of current upon washout of glibenclamide (see text).

(Fan and Makielski, 1997; Xie *et al*., 1999; Ribalet *et al*., 2000) and therefore faster at 37°C than at room temperature. In this experiment, the increased run-down drastically limited the degree of recovery. The current was ~95% inhibited by application of glibenclamide (3 nmol·L^{-1}) and was completely abolished by 100 nmol·L^{-1} glibenclamide. A minor current developed after a lag period during a 15 min washout. This current was abolished by reapplication of glibenclamide (100 nmol·L^{-1}), indicating that it was flowing through K_{ATP} channels. Subsequent washout for 25 min resulted in a slow recovery of ~5% of the initial current. In many cases, no recovery was observed during a washout period of >15 min consistent with control experiments (not shown) without glibenclamide that showed strong run-down that led to the complete loss of currents. The fraction of current recovered is a balance between run-down and the rate of channel reactivation. Glibenclamide dissociates slowly from wild-type channels (Figure 1), while at 37°C the rate of run-down is more pronounced. This increased rate and variable degree of run-down limited quantitative work with wild-type K_{ATP} channels at 37°C. However, the slow partial recovery with an initial lag phase (Figure 1B) observed with some cells suggest that the one site and graded models are unlikely to hold as they predict that the kinetics of glibenclamide dissociation and recovery of channel activity should coincide (see *Methods*, Eqn 4a) and should be fastest at the beginning of washout.

Experiments with the $K_{IR}6.2\Delta N5$/SUR1 channel

In the $K_{IR}6.2\Delta N5$/SUR1 channel, the N-terminal second through fifth amino acids of $K_{IR}6.2$ are deleted. This engineered channel was chosen as dissociation of glibenclamide was ~2.5-fold faster than from the wild-type (Figure 2A), thus reducing the time available for run-down; in addition, it has a higher open probability (Reimann *et al*., 1999), possibly rendering it less prone to run-down.

In five experiments, the dissociation rate constant, k_{-}(D), was determined as 0.13 ± 0.02 min^{-1}, corresponding to a half-time of dissociation, $t_{1/2}$(D) = 5.3 min. The association kinetics were determined at [^{3}H]-glibenclamide concentrations ranging from 0.89 to 3 nmol·L^{-1}; at [^{3}H]-glibenclamide concentrations >3 nmol·L^{-1}, apparent half-times were <2 min and the kinetics could not be monitored adequately by the slow filter assay. In the experimentally accessible concentration range, the apparent rate constant of association, k_{app}, increased linearly with [^{3}H]-glibenclamide concentration (Figure 2A and Eqn 2 in *Methods*) and, from the slope, the association rate constant was determined to be 0.30 ± 0.03 nmol·L^{-1}·min^{-1}. From these values, an equilibrium constant (K_D) was calculated to be 0.43 ± 0.05 nmol·L^{-1}. K_D was determined independently in [^{3}H]-glibenclamide saturation binding assays (not shown), which gave a value of 0.24 nmol·L^{-1} in fair agreement with the results of the kinetic experiments. Collectively, the data showed that the binding of glibenclamide to the truncated channel was in agreement with a simple bimolecular reaction mechanism and that glibenclamide dissociated from the channel with a dissociation rate constant k_{-}(D) = 0.13 min^{-1} ($t_{1/2}$(D) ~5.3 min).

In view of the faster dissociation of glibenclamide from the $K_{IR}6.2\Delta N5$/SUR1 channel, we tried to perform electrophysiological experiments in the inside-out configuration. Heating from 22°C to 37°C induced a strong run-down leading to an essentially complete loss of ATP-sensitive current in about 2–4 min (n = 10; not shown). This loss was independent of the presence or absence of activating solution (1 mmol·L^{-1} MgATP, 0.3 mmol·L^{-1} MgGDP). Further experiments were therefore performed in the whole-cell configuration at 37°C as currents were less affected by run-down. Figure 2 shows two example traces in which dialysis of the cell with the activating nucleotide solution induced a current that was abolished by application of glibenclamide (30 nmol·L^{-1}). At this concentration (~100 times the K_D value), the probability that all four sites of the channel were occupied by glibenclamide

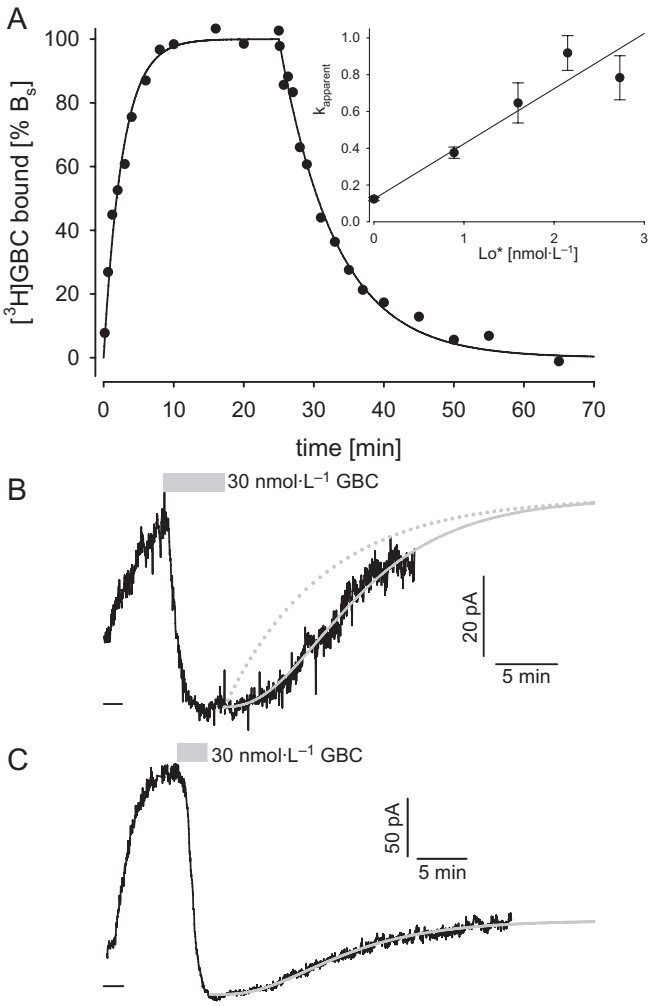

Figure 2 Experiments with $K_{IR}6.2\Delta N5/SUR1$. a. Association and dissociation kinetics of [^{3}H]-glibenclamide ([^{3}H]-GBC) in the presence of MgATP (1 mmol·L^{-1}) and MgGDP (0.3 mmol·L^{-1}). The association kinetics, measured in the presence of [^{3}H]-glibenclamide = 0.89 nmol·L^{-1}, gave k_{app} = 0.38 ± 0.03 min^{-1} and the dissociation kinetics initiated by addition of 30 nmol·L^{-1} glibenclamide (GBC) gave the dissociation rate constant, k_-(D), a value of 0.125 ± 0.009 min^{-1} corresponding to a half-time of 5.6 min. The inset shows the dependence of k_{app} on [^{3}H]-glibenclamide concentration (Lo*). From the fit of Eqn 2 to the experimental data, the association rate constant k_+ was determined to be 0.30 ± 0.03 nmol·L^{-1} min^{-1} and the equilibrium dissociation constant for glibenclamide binding to the channel was calculated to be 0.43 ± 0.05 nmol·L^{-1}. b and c. Inhibition of the $K_{IR}6.2\Delta N5/SUR1$ channel by glibenclamide and recovery from block (whole-cell recording at -60 mV). Glibenclamide (30 nmol·L^{-1}, applied for 5 min) abolished the current and washout for 15 min led to substantial recovery. The small bar at the beginning of the traces indicates the zero current level. b. The dotted line represents the exponential kinetics expected from the one-site model (Eqn 4c with k_-(D) = 0.12 min^{-1}) and the continuous curve, the fit to the four-site model (Eqn 4c giving k_-(R) = 0.16 min^{-1}). c. Fit to the four-site model gave k_-(R) = 0.14 min^{-1}.

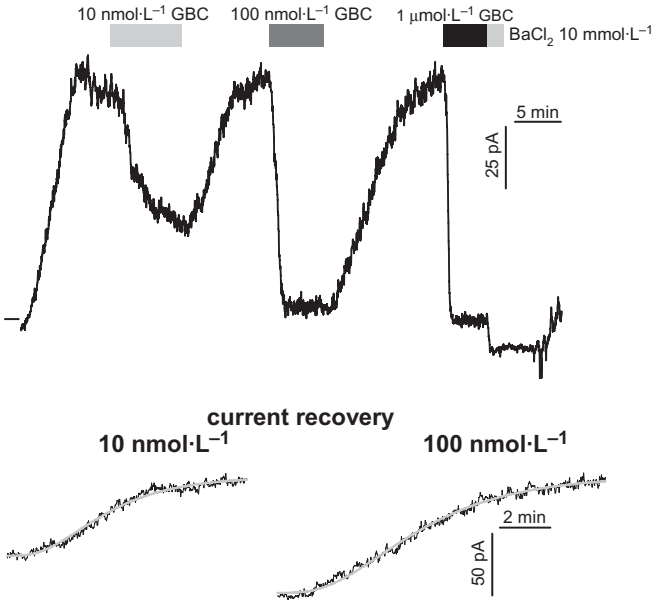

Figure 3 Inhibition of the $K_{IR}6.2\Delta N10/SUR1$ channel by glibenclamide (GBC) and recovery. Glibenclamide and Ba^{2+} were applied as indicated by the bars; conditions were as in Figure 2B,C; the small bar at the beginning of the trace indicates the zero current level. The close-ups show the recovery kinetics after washout of 10 and 100 nmol·L^{-1} glibenclamide and the fit of Eqn 4c, giving k_- values of 0.49 and 0.34 min^{-1} for 10 and 100 nmol·L^{-1} respectively.

approximated to 96%. Upon washout of glibenclamide, the current reappeared slowly after a lag phase. These kinetics are incompatible with the one-site model and graded models (exponential function; dotted curve in Figure 2B, see *Methods* Eqn 4a). Assuming that only the channel with all four SURs free of glibenclamide can reopen (four-site model) leads to Eqn 4c. This model gives a good fit to the prominent lag phase and sluggish time course of recovery yielding values of 0.16 and 0.14 min^{-1} for the dissociation rate constant of glibenclamide. The different extents of recovery reflect the variable degree of run-down as described above. In 17 experiments, seven showed <10% recovery during 20–30 min due to strong run-down and were not evaluated. In the remaining 10 experiments, recovery was 39 ± 6% of the current prior to application of glibenclamide and k_-(R) was 0.15 ± 0.02 min^{-1}, which is not different from the result of the kinetic experiments [k_-(D) = 0.13 min^{-1}, P = 0.551, t-test]. The half-time of recovery, $t_{1/2}$(R), was 12 min (Eqn 4d), which is 2.3 times higher than that of dissociation (Eqn 5).

Experiments with the $K_{IR}6.2\Delta N10/SUR1$ channel

With the $K_{IR}6.2\Delta N5/SUR1$ channel run-down was still a problem. We therefore examined the ΔN10 channel that shows a faster dissociation of [^{3}H]-glibenclamide (k_-(D) = 0.31 ± 0.01 min^{-1}, $t_{1/2}$(D) ~2.3 min; n = 3; not shown) and has a higher open probability (Reimann *et al*., 1999); in addition, we sought to learn more about how N-terminal truncation of $K_{IR}6.2$ affects the recovery kinetics of the channel. Figure 3 shows the response of this channel to 10, 100 and 1000 nmol·L^{-1} glibenclamide. The inhibition by 10 and 100 nmol·L^{-1} was readily reversible and followed a sigmoidal time course. The probability that all four sites of the channel were occupied was 82% and 98% respectively (K_D = 0.5 nmol·L^{-1}). Analysis of the recovery kinetics according to the four-site model (Eqn 4c) gave values of 0.49 and

Table 1 Parameters of recovery kinetics for a tetradimeric channel with *i* free sites required for full recovery of channel activity

i	*ΔN5*		*ΔN10*	
	$k_{-}(R)$ *min*$^{-1}$	y_{max} *(%)*	$k_{-}(R)$ *min*$^{-1}$	y_{max} *(%)*
4	0.15 ± 0.02	100	0.42 ± 0.03	100
3	0.11 ± 0.02	130 ± 13	0.36 ± 0.03	106 ± 1
2	0.061 ± 0.019*	250 ± 54	0.26 ± 0.03	120 ± 2[a]

Equation 6 in *Methods* was fitted to the traces obtained with the ΔN5 and ΔN10 channels ($n = 10$ and 6 respectively), giving the values for $k_{-}(R)$ and y_{max} (normalized with respect to the value for $i = 4$). For each curve, r^2 varied by less than 1% between models. $k_{-}(R)$ was compared with $k_{-}(D)$ (0.13 ± 0.01 and 0.31 ± 0.01 min^{-1} for the ΔN5 and ΔN10 channels respectively) by *t*-test. *$P < 0.05$.
[a]Not compatible with experimental traces (current after complete recovery significantly higher than before application of glibenclamide).

0.34 min^{-1} for the recovery rate constant $k_{-}(R)$ after inhibition by 10 and 100 nmol·L^{-1} GBC respectively. There was no difference in the kinetics of the recovery from 10 ($n = 3$) and 100 nmol·L^{-1} glibenclamide ($n = 3$) and results were pooled giving $k_{-}(R) = 0.42 \pm 0.03$ min^{-1} ($n = 6$), which was not different from the result of the dissociation experiments ($P = 0.551$). The half-time of recovery was 4.4 min, which is approximately twofold slower than the half-time of dissociation. Figure 3 also shows that 1 μmol·L^{-1} glibenclamide did not completely close the $K_{IR}6.2\Delta N10$/SUR1 channel (maximum inhibition 70 ± 5%; $n = 9$).

Modelling

The preceding analysis has shown that the four-site model is compatible with the data whereas the one-site model is not. Therefore, the compatibility of the intermediate stoichiometries ($i = 3$ or 2) with the data was also examined. Eqn 6 was fitted to the 10 traces obtained with ΔN5 and the six with ΔN10, letting $k_{-}(R)$ and the extent of recovery (y_{max}) float; the results are listed in Table 1. As judged by the overall quality of fit (given by the square correlation coefficient), the intermediate stoichiometries fit the curves as well as the four-site model. However, with decreasing *i*, good fits required the values for $k_{-}(R)$ to decrease and y_{max} to increase; that is, the curve was prolonged beyond the time range of the trace. For the three-site model, the changes in parameters were moderate and the $k_{-}(R)$ agreed even better with $k_{-}(D)$ than for the four-site model (Table 1). Also, the increase in the extent of recovery (+6%) was acceptable. For $i = 2$ ('half of the sites model'), $k_{-}(R)$ was significantly different from $k_{-}(D)$ (ΔN5 series, $P = 0.038$) and, in the ΔN10 series, the increase in the extent of recovery (+20 ± 2%) was incompatible with the experimental data (see for instance Figure 3). Therefore, this model is highly unlikely whereas the three- and the four-site models fit the experimental data equally well.

Discussion

The main results of this study are that at 37°C, recovery of the $K_{IR}6.2\Delta N$/SUR1 channels from inhibition by glibenclamide occurs on a time scale amenable to electro-physiological experiments, that it follows a sigmoidal time course and that a relationship can be established between the dissociation kinetics of glibenclamide and the kinetics of channel recovery. The data are in quantitative agreement with (but do not necessarily prove) models where three or all four subunits must be free of glibenclamide before the channel can reopen; models where dissociation of one or two glibenclamide molecules leads to appreciable channel activity are highly unlikely (two sites) or can be excluded (graded and one site models).

Assumptions and limitations

We considered the possibility that the sigmoidal recovery kinetics might arise from slow washout of glibenclamide from the cell or by diffusion in and out of the pipette. Experiments in the inside-out configuration where such problems might be minimized (but see Nichols *et al.*, 1991) were not possible due to rapid channel run-down upon heating to 37°C. However, amphiphilic inhibitors with weaker affinity show unblocking of K_{ATP} channels in the whole-cell configuration with a time course of 1–5 min and without apparent sigmoidicity. For example, the unblocking of $K_{IR}6.1$/SUR2B from PNU96296 (Lange *et al.*, 2002) and $K_{IR}6.1$/SUR2B from the glibenclamide congener, HMR 1883 (100 μmol·L^{-1}; Russ *et al.*, 2001), are both rapid processes. These examples imply that slow washout and diffusion are not an intrinsic problem in the experiments and that the slow dissociation of glibenclamide and the stoichiometry of unblocking SUR1-containing channels underlie the slow sigmoidal kinetics.

A central assumption in the modelling is that the recovery kinetics are dominated by the dissociation of the ligand and that any subsequent conformational changes of the channel are not rate-limiting. We assume that any conformational changes are independent of the particular sulphonylurea used. The recovery from tolbutamide, a sulphonylurea with much weaker affinity for SUR1, is rapidly reversible (see Ashfield *et al.*, 1999), thus any conformational changes must be 'fast'.

Truncated versus wild-type channels

A major question is whether the observations with the N-terminal truncated channels can be extrapolated to wild-type $K_{IR}6.2$/SUR1 channels. Experimentally, the extent of channel recovery following washout of glibenclamide is determined by a competition between channel reactivation versus run-down, which is rapid at 37°C. This strongly limits the fractional recovery of wild-type channels with their slow rate of glibenclamide dissociation. However, we sometimes observed sluggish recovery kinetics, incompatible with the one-site and the graded model. To support these limited observations, we used channels with N-terminal truncated $K_{IR}6.2$ subunits that are well characterized and show a faster dissociation of glibenclamide (half-times 2.3 and 5.3 min for the ΔN5 and ΔN10 channels vs 14 min for wild type), thus favouring channel reactivation over run-down at 37°C. The recovery kinetics of the truncated channels were sigmoidal with half-times twofold or more than twofold slower than the half-times of dissociation.

Progressive N-terminal truncation of $K_{IR}6.2$ increases the open probability of the channel by decreasing the time the channel spends in the long-lived closed state (Babenko *et al.*, 1999a; Koster *et al.*, 1999b; Reimann and Ashcroft, 1999). These channels were less prone to run-down perhaps due to a decreased dependence on phoshoinositides for activity. On the other hand, stabilization of the long-lived closed state is one of the inhibitory mechanisms of the sulphonylureas and N-terminal truncation of $K_{IR}6.2$, therefore interferes with the ability of sulphonylureas to inhibit the channel. Under the activating conditions used here, glibenclamide is approximately fivefold less potent on the ΔN5 channel versus wild type, but only reduces the maximal inhibition from 100% to 98% (R. Prager and U. Quast, unpubl. results). With the ΔN10 channel under activating conditions, the maximal inhibition of channel activity by glibenclamide was decreased by 30% and the potency by ~200-fold (IC_{50} = 10 nmol·L^{-1}; R. Prager and U. Quast, unpublished), although the affinity of glibenclamide binding was only slightly reduced (K_D = 0.5 nmol·L^{-1}). In the absence of activating nucleotides, the potency of tolbutamide on the ΔN5 channel is decreased approximately fivefold and maximal inhibition by 30% (corrected for the incomplete inhibition by glibenclamide in inside-out patches; Reimann *et al.*, 1999). The ΔN10 channel is only marginally (Babenko *et al.*, 1999b) or not at all (Reimann *et al.*, 1999) inhibited by sulphonylureas, showing that in this channel, glibenclamide could not induce the transition to the long-lived closed state.

The loss of this inhibitory mechanism (in the ΔN10 channel) or its presence (in the ΔN5 channel) did not affect the basic characteristics of the recovery kinetics, that is, the sigmoidal time course in quantitative agreement with the four- and three-site models. It is therefore plausible to assume that these characteristic traits hold for the wild-type channel, which has a lower open probability than the ΔN5 channel and is thus more sensitive to inhibitors such as glibenclamide and ATP that stabilize the long-lived closed state. The limited data available for the wild-type channel support this conclusion.

Stoichiometry of recovery from inhibition

The recovery kinetics were compatible with both the four- and three-site stoichiometries. Independent support for the four-site model comes from Schwanstecher's group who compared binding of sulphonylureas to SUR1 with inhibition of the $(K_{IR}6.2/SUR1)_4$ channel (Dörschner *et al.*, 1999). They found that the sulphonylurea inhibition curves were shifted to 5.5-fold lower concentrations versus the binding curves and that the Hill coefficient of the inhibition–concentration curve was 1.27. Both observations suggest a mechanism where binding of one glibenclamide is sufficient to inhibit channel activity, which implies that all sites must be free for the channel to open (four-site model). We note that the shift of the binding versus the inhibition curves is based on binding of glibenclamide to SUR1 alone (Dörschner *et al.*, 1999), while coexpression with $K_{IR}6.2$, to assemble K_{ATP} channels, enhances the affinity of SUR1 for sulphonylureas in general, and glibenclamide in particular, three to fourfold (in cells: Winkler *et al.*, 2007; in membranes: Hansen *et al.*, 2005). Therefore, much of the curve shift observed by Dörschner *et al.* (1999) reflects the fact that $K_{IR}6.2$ was absent in the binding studies and that $K_{IR}6.2$ contributes to the sulphonylurea binding site of the channel (Vila-Carriles *et al.*, 2007; Winkler *et al.*, 2007). For the wild-type channel, we independently determined a 10-fold leftward shift of the inhibition versus the binding curve (Stephan *et al.*, 2006), which largely accounts for the one-site inhibition stoichiometry proposed by Dörschner *et al.* (1999).

Further support for the idea that occupation of a single inhibitory site, either on SUR1 or $K_{IR}6.2$, closes the tetradimeric channel is the unambiguous demonstration that occupation of one inhibitory ATP site on the $K_{IR}6.2$ tetramer induces channel closure (Markworth *et al.*, 2000). Conversely, using the $K_{IR}6.2$(F331I) mutation, which enhances channel activation by MgATP via SUR1 and causes permanent neonatal diabetes mellitus, Tammaro *et al* (2005) showed that at least three SUR subunits must bind/hydrolyse MgATP to overcome channel inhibition by MgATP binding to $K_{IR}6.2$. This result is in complete agreement with the stoichiometry of recovery from inhibition by glibenclamide.

In conclusion, we have shown that the recovery from block by glibenclamide of tetradimeric channels formed from SUR1 and N-terminally truncated $K_{IR}6.2$ subunits, ΔN5 or ΔN10, is reversible and follows a sigmoidal time course. The recovery kinetics together with evidence that a single sulphonylurea can block the channel favour a model that requires dissociation of glibenclamide from all four SUR subunits before the channel can regain activity. It is very likely that these results also hold for the wild-type channel.

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Conflicts of interest

The authors state no conflict of interest.

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