Kinetics of drug interaction with the Kv11.1 potassium channel

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Non-standard abbreviations: hERG – human ether a-go-go related gene, Kv – voltage-gated potassium channel, I_Kr – rapid component of the delayed rectifier current, aLQTS – acquired long QT syndrome
Abstract

The \textit{Kv11.1} potassium channel is the molecular target for the majority of drugs implicated in acquired long QT syndrome (aLQTS), the commonest cause of drug-induced sudden cardiac death, and a common reason for drug restriction or withdrawal from the market. Whilst the IC\textsubscript{50} for block of Kv11.1 is commonly used to estimate the risk of aLQTS, this approach is crude and it is widely accepted that the kinetics of drug interactions with the channel are a critical component in understanding their mechanism of action and risk profiles. In this study we report the first directly measured kinetics of block and unblock of Kv11.1 by a QT prolonging drug – the antipsychotic clozapine. Our data show that clozapine binding to Kv11.1 is complex. There are at least two kinetically distinct components to both block and unblock while the kinetics of unblock are dependent on the dose or duration of drug application. Based on these observations we have proposed a model incorporating kinetically distinct binding to the open and inactivated states of Kv11.1 that can describe the observed kinetic features of clozapine block and correctly predict the overall affinity and apparent non-state dependent interaction of clozapine with Kv11.1. Mechanistic insights into drug block of Kv11.1, gained though detailed kinetic analysis such as this, have a potential role in development of drugs targeted to specific channel states to reduce unwanted side effects as well as in the design of better high throughput preclinical tests for assessing the proarrhythmic effects of QT prolonging drugs.
Introduction

The Kv11.1 channel (often referred to as the human ether à-go-go related gene (hERG) K⁺ channel) is the pore forming subunit of the rapid delayed rectifier potassium current (I$_{Kr}$) (Sanguinetti et al., 1995). I$_{Kr}$ ensures timely repolarization of the cardiac action potential and opposes the propagation of premature ventricular beats (Smith et al., 1996; Lu et al., 2001). Kv11.1 channels are also the molecular target for a wide range of drugs associated with an increased risk of a life-threatening form of arrhythmia known as torsades de pointes (TdP) (Wood and Roden, 2004). For example, 1-3% of patients with schizophrenia on various antipsychotics will have ‘prolonged QT’ at some time (Sanguinetti et al., 1995; Lieberman et al., 2005), while 3 deaths per 1000 patient years occur for treated schizophrenia (Smith et al., 1996; Lu et al., 2001; Ray et al., 2009). Consequently, measured Kv11.1-drug affinity is a core component of regulatory guidelines for the pre-clinical investigation of a drug’s propensity to prolong the QT interval (ICH S7A and S7B, http://www.ich.org). In this regard, based on a literature survey of 100 drugs it has been concluded that if the IC$_{50}$ of a drug for block of Kv11.1 is more than 30-fold higher than the effective therapeutic plasma concentration then the drug should be safe (Redfern et al., 2003; Wood and Roden, 2004).

Whilst such a criterion is likely to exclude virtually all proarrhythmic drugs, it also runs the risk of excluding drugs that may not be problematic. Measurements of IC$_{50}$ are only a crude estimate of how block of Kv11.1 channels will affect cardiac repolarization. For example, drugs that have slow binding and unbinding kinetics have the paradoxical effect of showing reverse use dependence, i.e. they cause more significant prolongation of cardiac repolarization at slow heart rates (due to the complex interplay between the kinetics of Kv11.1 gating and the effect of heart rate on repolarization reserve) whereas drugs with more rapid binding and unbinding kinetics can dissociate during long diastolic intervals and so
cause less block at slow heart rates (Roden and Hoffman, 1985). The potential therefore exists for a small number of potentially useful, and ultimately safe, medications to be withdrawn from development for failing pre-clinical QT studies. To avoid this possibility, we need to develop a better understanding of the intricacies of how drugs interact with the Kv11.1 channel.

It is well established that drug block of Kv11.1 can be voltage- (Paul et al., 2002; Witchel et al., 2004; Kamiya et al., 2008) and state-dependent (Perrin et al., 2008) and these properties may vary substantially from drug to drug (Perrin et al., 2008). Consequently the apparent IC₅₀ can vary considerably depending on the voltage protocol used to measure block (Kirsch et al., 2004; Milnes et al., 2010). Furthermore, extracting kinetic parameters for drug binding and unbinding to Kv11.1 is complicated by the unusual gating kinetics of this channel (Vandenberg et al., 2012). To our knowledge there is no study that has directly measured the kinetics of both binding and unbinding of drugs from the Kv11.1 channel. In addition to providing insights into the mechanism of drug binding, knowing the kinetic scheme of drug interaction is essential for formulating in silico models. Such models can then be used to study the effect of drugs on cardiac electrical activity and investigate the factors that define the proarrhythmic propensity of individual drugs.

For this study we have used clozapine (8-chloro-11-(4-methyl-1-piperazinyl)-5H-dibenzo(b,e)(1,4)diazepine) to gain insights into the kinetics of drug binding and unbinding to Kv11.1. Clozapine was chosen for two reasons. First, it is a commonly used anti-psychotic agent that has been associated with acquired long QT syndrome (Ray et al., 2009). Second, and more importantly from a practical perspective, the kinetics of washoff are relatively rapid (on the order of 10s of seconds) (Lee et al., 2006). This is important for patch clamp studies...
where current rundown and deterioration of patch quality occurs on the order of 10-15 minutes. We found that clozapine block and unblock of Kv11.1 channels has surprisingly complex kinetics whereby the rate of unbinding is dependent on the dose (higher doses have slower washout) and duration of exposure (slower washout after longer exposure). Based on these observations we have proposed a kinetic scheme that is able to reproduce the kinetic features of clozapine block and unblock of Kv11.1 as well as correctly predict the overall efficacy of block of both WT and inactivation deficient Kv11.1.
Materials and Methods

Molecular Biology

Experimental data was acquired from Chinese hamster ovary (CHO) cells transfected with either wild type (WT), or N588K Kv11.1 channel cDNA using Lipofectamine LTX plus reagent (Life Technologies) as per manufactures instructions. CHO cells were cultured in Dulbecco’s modified Eagle’s medium / F-12 (Invitrogen) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 1x non-essential amino acids (Invitrogen, Australia), 1x GlutaMAX (Invitrogen, Australia), and maintained at 37°C in 5% CO₂.

Electrophysiology

Glass capillary patch electrodes with resistance of 2–4 MΩ, when filled with internal solution, were made using a vertical two-stage puller (PP-830, Narishige, Tokyo, Japan). The internal solution contained (in mM): 120 potassium gluconate, 5 EGTA, 10 HEPES, 20 KCl, 1.5 Mg-ATP, pH 7.3 with KOH. Standard external solution contained (in mM): 1 MgCl₂, 1 CaCl₂, 10 HEPES, 12.5 Glucose, 5 KCl, 130 NaCl, 0.1% dimethyl sulfoxide (DMSO), pH 7.4 with NaOH. The calculated junction potential for these solutions was -15 mV. Cells were voltage-clamped in whole cell configuration using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). Current signals were digitized at 5 kHz, filtered at 1 kHz and stored on an IBM-compatible PC interfaced with a Digidata 1440A analogue-digital convertor (Molecular Devices). Series resistance was compensated by at least 80% in all experiments.

Pharmacology

Clozapine was purchased from Sigma-Aldrich (Australia) and prepared as a stock solution in DMSO before dilution as required in external solution such that the maximum final DMSO concentration was 0.1% v/v. It has been reported previously that DMSO at 0.1% (v/v) has no
effect on gating properties of Kv11.1 channels (Walker et al., 1999). To measure drug block, cells were depolarized to 0 mV for 10 seconds to ensure channels were fully activated (i.e. open or inactivated). Based on our previous studies, we calculated that at room temperature, Kv11.1 channels expressed in CHO cells would be 99.9% activated at 0 mV (Vandenberg et al., 2006). The current at 0 mV was then continuously monitored as drugs were applied and removed using the Dynaflow Resolve system (Cellectricon, Mölndal, Sweden) to obtain kinetics of washon and washoff. The Dynaflow Resolve System is a microfluidic device that uses laminar patterned flow to maintain discrete solutions (with e.g. different concentrations of drugs) within a bulk aqueous system (Olofsson et al., 2004); see Supplementary Figure 1A). In our hands, the solution exchange time for the Dynaflow Resolve System was 26 ± 1 ms (see Supplementary Figure 1B) allowing very accurate measurement of the kinetics of block and unblock following solution exchange.

Drug binding models

Variations on a simplified Markov model of drug binding to Kv11.1 that included open and inactivated states as well as an open state bound to drug (OD), and an inactivated state bound to drug (ID) (Perrin et al., 2008) were used to describe the experimental data. Models were fitted to an idealized dataset derived from an average of 4 experimental data traces acquired as described in Figure 3A. Parameter estimation was completed using the Optimization Toolbox component of the MATLAB package (Mathworks, MA, USA). Where relevant, microscopic reversibility was ensured by imposing equality of the products of the forward and reverse transition rates of closed loops (Colquhoun et al., 2004). Multiple optimization runs from different starting parameters were completed to confirm the identified ‘best fit’ parameter sets.
Dissociation constants (K$_d$) for drug interactions with individual states of the channel were calculated from the optimized rate constants such that:

$$K_d = \frac{k_b}{k_f}$$

Where $k_f$ and $k_b$ are the forward and reverse rate constants respectively.
Results

Block of Kv11.1 channels by clozapine

Typical currents recorded at 0 mV from a cell expressing Kv11.1 channels during 20 s exposure to 1, 3 and 10 µM clozapine are shown in Figure 1A. The extent of block, measured at the end of the 20s drug application, was used to estimate the IC₅₀ for clozapine block of Kv11.1 channels. The mean data for the dose response curves are shown in Figure 1B. The calculated IC₅₀ for clozapine block of WT Kv11.1 channels based on the 20s drug application data is 2.8 ± 0.3 µM (SEM; n=5). An important observation from the current traces shown in Figure 1A is that the kinetics of drug block of Kv11.1 channels are quite complex. For example, in response to application of 3µM clozapine the timecourses of both block and unblock are clearly not monophasic. We therefore undertook a more detailed analysis of the kinetics of drug binding.

In Figure 2, currents recorded at 0 mV during 20 s application of different doses of clozapine ranging from 1 to 20 µM are plotted superimposed (Figure 2A). The same set of data are replotted in Figure 2B but this time normalized for the maximum observed block for each drug concentration. As expected, the observed rate of current block increases as the dose of clozapine increases. However, what was not expected was that the rate of current recovery during washout of higher doses of clozapine was clearly slower than the rate of washout after lower doses. For a simple bimolecular reaction:

\[
D + R \rightleftharpoons DR
\]  

(1)

where D is drug and R is receptor, the rate of current recovery following drug washout should be independent of the dose of drug that was added (Hille, 2001). Furthermore, it is also clear that there are two components to both the block and unblock phases (arrowheads in Figure 2A highlight the transition from the fast to the slow phase of current recovery) as opposed to
the monophasic block and unblock profiles that would be expected from a bimolecular reaction (Hille, 2001). These observations clearly demonstrate that clozapine block of Kv11.1 channels is incompatible with a simple bimolecular reaction scheme.

To further investigate the two components of drug block and unblock of Kv11.1 channels we examined whether adding the same dose of drug for different periods of time affected the kinetics of block and unblock. A typical current trace recorded at 0 mV showing block (highlighted red) and unblock (highlighted blue) of Kv11.1 current in response to varying durations of washon of 5 µM clozapine (ranging from 0.25 s to 40 s) is shown in Figure 3A. In Figure 3B we have superimposed the washon phases for each different duration of drug application, which overlap with each other as expected. In Figure 3C we have superimposed the unblock phases acquired during drug washoff, which in contrast to the blocking phase, are clearly very different to each other. Specifically, the rate of unblock of current during washoff is slower after longer applications of drug. In this example, the timecourse for current recovery after the shortest drug application (0.25 s) was dominated by a fast component with a time constant of 608 ms and after the longest drug application (40 s) current recovery was dominated by a slow component with a time constant of 15.1 s (Figure 3D).

In Figure 4 we have plotted the current trace corresponding to block and unblock of current for a 10 s application of 5 µM clozapine both with a normal current scale (Figure 4A) and also on a logarithmic scale (Figure 4B) to highlight the two distinct phases for both the washon and washoff. The time constants obtained by fitting biexponentials to each phase were $0.4 \pm 0.01$ s and $5.1 \pm 0.1$ s for the fast and slow components of washon and $0.7 \pm 0.04$ s and $16.3 \pm 1.6$ s for the fast and slow components of washoff (SEM; n=4-5, Figure 4C). We
next investigated whether the kinetics of unblock following all the different durations of drug application could be described as the sum of the same two time constants obtained from the fits to the 10 s data traces (summarized in Figure 4C). Not only was it possible to fit all data traces with a biexponential function described by these two time constants (Figure 5A), but the magnitude of the slow component increased (and the fast component decreased) as the duration of drug application was increased (Figure 5A-B). Furthermore, the time constant of the single exponential fit to the relationship between washon duration and the amplitude of the slow component of unblock in Figure 5B was 5.0 s which corresponds to the slow component of the washon phase shown in Figure 4 (5.1 ± 0.1 s). This suggests that the slow component of block is related to the slow component of unblock.

The analysis above suggests that there are at least two distinct binding sites for clozapine in the Kv11.1 channel. We therefore investigated whether we could reproduce this data using a simple Markov scheme. As our recordings were made from fully activated channels, i.e. they may exist in open or inactivated, but not closed states, the simplest model we could start with was one that involved four states: open (O) and inactivated (I) as well as drug-bound open (O-D) and drug-bound inactivated (I-D) states. We first considered a scheme that allowed transitions between all four states (see Figure 6Ai). In this model the rate constants for inactivation and recovery from inactivation were fixed according to previously published measurements (Vandenberg et al., 2006) whilst the other rate constants were obtained by fitting the model to an idealized data trace derived from the average of n=4 experiments acquired as described in Figure 3A. Multiple runs of optimization from different starting parameters identified a common parameter set that best described the data (Mean rate parameters from n=5 optimization runs are summarized in Figure 6B). Interestingly, in this model the transition rate between the O-D and I-D states was effectively zero, i.e. more than
6 orders of magnitude slower than the O-I transition. Furthermore, fitting of an alternative model with no transitions between O-D and I-D yielded very similar rate parameters to those summarized in Figure 6Aii (Supplementary Figure 2A). We also considered a scheme with no drug binding to the inactivated state (i.e. where transitions between I and I-D were not permitted) but rather occupation of the I-D state could only occur via the O-D state. This scheme also provided very good fits to the data (Supplementary Figure 2B). Since we currently have no experimental data with which to discriminate between the different kinetic schemes we used the full model shown in Figure 6Ai, that includes all state transitions, for further considerations.

From the model presented in Figure 6A we can calculate $K_d$ values for drug binding to the open and inactivated states (since O-D to I-D transitions are negligible). These calculated $K_d$ values were very similar to each other (1.46 and 1.63 µM respectively) as well as to the experimentally determined IC$_{50}$ value for clozapine block of Kv11.1 (2.8 ± 0.3 µM, Figure 1B). It is important to note that the experimental IC$_{50}$ curves were not used to constrain the modeling so in this context the model predictions and the experimental traces are in good agreement. Based on the similarity of the calculated $K_d$ values for drug binding to the open and inactivated states, our model predicts that Kv11.1 mutations that affect the distribution of open versus inactivated states should not affect the experimentally determined IC$_{50}$ for clozapine. We tested this hypothesis using N588K Kv11.1 channels. At 0 mV, the distribution of open and inactivated states in WT Kv11.1 channels will be ~5:95 whereas in N588K Kv11.1 channels it will be ~95:5 (Perrin et al., 2008). Consistent with our model predictions the IC$_{50}$ for clozapine block of N588K Kv11.1 channels was very similar to that for WT Kv11.1 (3.2 ± 0.3 and 2.8 ± 0.3 µM respectively, Figure 7). We attempted to fit the model in Figure 6 directly to the N588K data solely by changing the rate constants for
inactivation and recovery from inactivation but leaving the drug binding rates unchanged. This however did not provide good fits to the data (data not shown). This suggests that the N588K mutation may subtly affect access to the drug binding pockets (but not the overall affinity).
Discussion

In this study, we have provided the first direct measurement of the kinetics of drug binding to and unbinding from Kv11.1 channels. Our data show that for clozapine there are at least two kinetically distinct components to drug block and unblock, which we suggest represent binding to the open and inactivated states of Kv11.1 channels. This hypothesis is consistent with the well-described phenomenon of state-dependence of drug block of Kv11.1 channels (Perrin et al., 2008). Interestingly, the calculated affinities for clozapine binding to the open and inactivated states, based on our modeling data, were very similar. Thus whilst there may be two distinct binding sites they are not separable based on steady-state or equilibrium measurements. In support of this prediction, we found that the experimentally measured IC$_{50}$ values for clozapine block of WT and the inactivation deficient mutant, N588K were very similar.

Understanding the kinetics of drug block of Kv11.1 channels has two important outcomes. First, this data is essential for constraining Markov state models for drug binding to Kv11.1 channels that can be incorporated into cardiac myocytes and ultimately whole heart models of cardiac electrical activity to assess the risk of drug-induced QT prolongation and cardiac arrhythmias. Second, it can help explain the protocol dependence of measured IC$_{50}$ values (Di Veroli et al., 2013b) and perhaps more importantly enable one to design more effective assays for assessing drug binding to Kv11.1 channels as part of pre-clinical testing of QT prolonging drugs. The approach employed in this study that has made detailed kinetic characterization of block tractable is the very fast, programmable solution switching that is possible using the microfluidic Dynaflow Resolve system (Cellecrticon, Sweden). This system allows solution exchange on the order of ~20 ms meaning 1) Current block and recovery in response to very short and well-defined washon durations (as low as 250 ms in
this study) can be measured and 2) Fast components of block or unblock (of the order of 100’s milliseconds in this study), that could potentially be missed with slower solution exchange yet may be critical for understanding the mechanism of block, can be resolved. Indeed, it is precisely this latter point that allowed us to make the observations that formed the basis of our kinetic model.

Our data demonstrate that discussion of the state-dependence of drug binding based on equilibrium measurements as opposed to kinetic criteria is potentially misinformed. For example, it has long been suggested that drug binding to Kv11.1 channels may show state dependence (Ficker et al., 1998). In 2008, based on steady-state measurements of drug block of WT and inactivation deficient mutants, we suggested that some drugs showed state-dependence of binding whilst others do not (Perrin et al., 2008). According to these criteria, clozapine would be classified as not showing state-dependence of binding since the measured IC₅₀ for WT and the inactivation deficient N588K Kv11.1 mutant were not significantly different (Figure 7). What is clear from our study, is that clozapine shows two kinetically distinct blocking effects, consistent with differential binding to the open and inactivated states. However, the affinity for each of these two distinct states is very similar and so can only revealed by kinetic measurements. While it remains to be seen how these observations translate to other drugs, we would suggest that greater clarity in our understanding of these concepts will be helpful in advancement of the field since the notion of state-dependent binding is so interwoven into our discussion of high affinity block of Kv11.1 (Weerapura et al., 2002; Perrin et al., 2008) and its relationship to proarrhythmia .

The importance of considering kinetics in understanding the proarrhythmic properties of drugs is a widely accepted concept, and many groups have used kinetic descriptions of
binding in their in silico analyses (Brennan et al., 2009; Zhou et al., 2009; Saiz et al., 2011; Di Veroli et al., 2013b). However, none of these studies have directly measured the kinetics of block and unblock, but rather inferred kinetics from more complex pulsing protocols. Only by directly measuring the kinetics of drug interactions with the channel were we able to obtain the data necessary to constrain the transition rates in our model of binding to the open and inactivated states. Our model reproduces all the main characteristics of clozapine interaction with the wildtype Kv11.1 channel including the biphasic nature of both block and unblock, the washon duration-dependence of the amplitude of the slow component of unblock, and the similar affinities of clozapine for the open versus inactive states. In the pursuit of an in silico approach to assessing the proarrhythmic propensity of QT prolonging drugs this type of detailed kinetic constraint is critical in working towards a unified model. There are multiple different model structures that have been proposed for drug interaction with Kv11.1 (Zhou et al., 2009; Saiz et al., 2011; Di Veroli et al., 2013b), that at least in part likely stems from the variety of relatively complex protocols used to obtain the data that constrains them. We would argue that our back to basics approach, of directly measuring the block and unblock without complex pulsing protocols allows development of models at the fundamental level, which can be further refined in future with more complex experimental datasets that for example included activation and deactivation as well as inactivation.. Based on these foundations provided by the present study, the long-term aim of our studies is to build a complete kinetic model of drug block of Kv11.1 channels that can be incorporated into computer models of cells and ultimately whole hearts. Before we can do this though we will need to include (i) the voltage dependence of the kinetics of drug binding to the open and inactivated states (Carmeliet, 1992), and the (ii) kinetics of binding or dissociation from the closed state (Mitcheson et al., 2000), which has been demonstrated for some compounds (Milnes et al., 2003). We also need to verify that our model can apply to a wide range of
drugs including those that show apparent differences in binding to open versus inactivated states as well as those that show dissociation from closed states.

Limitations

One of the limitations of any kinetic analysis of drug block is the lag time associated with drug application, and its washoff. For the Dynaflow Resolve system in our hands the lag time ranges from ~10-40 ms. This clearly will be insignificant when considering the slow time constants in our experiments but it is approaching 10% of the values for our measured fast time constants. When considering drugs with an even lower affinity than clozapine and so faster time constants of unblock, then it may not be possible to get accurate estimates of the fast components unless we explicitly include a drug diffusion term in the model.

Conclusions

It is widely accepted that an understanding of the kinetics of drug interactions with the Kv11.1 channel is fundamental to our insight into the proarrhythmic propensity of these drugs (Di Veroli et al., 2013a). In this study we have provided the first direct measurement of the kinetics of drug binding to unbinding from Kv11.1 channels at high temporal resolution. We show that for clozapine, both block and unblock are biphasic processes and that the kinetics of unblock are dependent on the dose or duration of drug application – observations that are inconsistent with simple bimolecular interactions. We suggest that these phenomena can be explained by differential binding to the open and inactivate states of the channel. Based on these findings we have proposed a model that can describe the kinetic features of clozapine interaction with Kv11.1 and is able to correctly predict the similar experimentally measured IC50 values for both WT and inactivation deficient KV11.1. The insights into the mechanism of drug block of Kv11.1 gained though detailed kinetic analysis, such as that
presented here, has potential to help in development of drugs targeted to specific states of the channel to reduce unwanted side-effects as well as in the design of better assays for assessing the proarrhythmic effects of QT prolonging drugs in preclinical testing.

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Authorship Contributions

*Participated in research design:* Hill, Perrin, Campbell, Vandenberg.

*Conducted experiments:* Hill, Heide.

*Performed data analysis:* Hill, Mann, Vandenberg.

*Wrote or contributed to the writing of the manuscript:* Hill, Perrin, Mann, Vandenberg
References


Footnotes

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Figure Legends

Figure 1: Clozapine block of Kv11.1. A) Typical examples of clozapine block of Kv11.1 after depolarization to 0 mV. Data is shown for 1, 3 and 10 μM clozapine. For each concentration, block was measured after 20 s of application (open circles). B) Dose dependent block of Kv11.1 by clozapine. A fit of the Hill equation to the data yielded an IC50 of 2.8 ± 1.0 μM (SEM; n=5).

Figure 2: Clozapine block of Kv11.1 has complex kinetics. A) Typical examples of block of Kv11.1 at 0 mV. Each dose of clozapine was applied for 20 s (black bar) before washoff. Filled arrows define the transition from the fast to slow component of unblock. B) Data from (A) normalized to the maximum observed block highlighting the dose dependent kinetics of both block and unblock.

Figure 3: Measuring the kinetics of clozapine block of Kv11.1. A) A typical example of the protocol used to measure the kinetics of clozapine block (highlighted red) and unblock (highlighted blue) of Kv11.1. Cells were depolarized to 0 mV before 5 μM clozapine was applied for time periods ranging from 250 ms to 40 s (red bars). Complete washoff was measured after each application. B) Timecourses of block from each of the washon durations from (A) superimposed to demonstrate consistency in kinetics of block. C) Unblock phases following each of the washon durations from (A) superimposed to highlight the dependence of the kinetics of unblock on the duration of drug application. D) Single exponential fits to the major components of unblock following the shortest (250 ms) and longest (40 s) washon durations of clozapine.

Figure 4: Clozapine block and unblock of Kv11.1 are biphasic processes. A) Typical
example of clozapine block of Kv11.1 at 0 mV. Clozapine, 5 µM, was applied for 10 s before washoff. Washon and wasoff phases are highlighted in red and blue respectively. B) Data from A replotted on a logarithmic scale to highlight the biphasic nature of both block and unblock. Data shown corresponds to the boxed regions in A. Fits to both the fast and slow components of block (τ_{on,f} and τ_{on,s}) and unblock (τ_{off,f} and τ_{off,s}) are shown (dashed lines). C) Time constants describing the fast and slow components of block and unblock. Data are Mean ± SEM; n=4.

**Figure 5: Washon duration defines the kinetics of unblock.** A) Examples of unblock timecourses following washon of clozapine, 5 µM, for (i) 500 ms and (ii) 20 s. Each example of unblock was fitted with a different mix of the same two time constants identified in Figure 4C (red dash lines). The amplitude of the slow component of unblock increased after longer applications of drug (92 % versus 24 % after 20 s and 500 ms washon durations respectively). B) Relationship between washon duration and amplitude of the slow component of unblock. A single exponential fit to the data yielded a time constant of 4960 ms. Data are mean +/- SEM; n=4.

**Figure 6: Kinetic model of clozapine block of Kv11.1.** Ai) Four state Markov model describing drug binding to open and inactive states of Kv11.1. ii) Parameter set derived from fitting of the Markov model in (i) to an idealized experimental dataset derived from average of n=4 experiments acquired as described in Figure 3A. B) Overlay of simulated drug block (red line) with the most similar individual experimental dataset. Data shown are for 500 ms, 5 s and 20 s washon durations. Filled red arrows define the transition from the fast to slow component of unblock for the simulated data. C) Comparison of simulated (red line) and experimental (grey) data describing dose dependent block of Kv11.1 by clozapine. IC_{50} for...
simulated data was 1.8 μM versus 2.8 ± 1 μM for experimental datasets.

**Figure 7: Clozapine block of inactivation deficient N588K Kv11.1.** A) Typical examples of block of N588K Kv11.1 at 0 mV. Each dose of clozapine was applied for 20 s (black bar) B) Dose dependent block of N588K Kv11.1 by clozapine. A fit of the Hill equation to the data yielded IC$_{50}$ of 3.2 ± 0.3 μM for N588K (Black triangles, SEM; n=4) compared to 2.8 ± 1 μM for WT Kv11.1 (Grey circles).
FIGURE 1

A

B

$\frac{I_{\text{drug}}}{I_{\text{control}}}$ vs. log [clozapine] (M)

current (pA)

0 mV

1 µM 3 µM 10 µM

20 s
FIGURE 3

A

B washon

C washoff

D

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FIGURE 6

Ai

\[
\begin{align*}
O & \rightarrow I \\
D & \rightarrow O \\
O-D & \rightarrow I-D
\end{align*}
\]

ii

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B

C

\[
\left| \frac{I_{\text{drug}}}{I_{\text{control}}} \right|
\]

\[
\text{log [clozapine] (M)}
\]
FIGURE 7

A

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