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Kinetic Analysis of the Inhibitory Effect of Glibenclamide on K_{ATP} Channels of Mammalian Skeletal Muscle

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Abstract. We investigated the block of K_{ATP} channels by glibenclamide in inside-out membrane patches of rat flexor digitorum brevis muscle.

(1) We found that glibenclamide inhibited K_{ATP} channels with an apparent K_i of 63 nM and a Hill coefficient of 0.85. The inhibition of K_{ATP} channels by glibenclamide was unaffected by internal Mg^{2+}.

(2) Glibenclamide altered all kinetic parameters measured; mean open time and burst length were reduced, whereas mean closed time was increased.

(3) By making the assumption that binding of glibenclamide to the sulphonylurea receptor (SUR) leads to channel closure, we have used the relation between mean open time, glibenclamide concentration and K_D to estimate binding and unbinding rate constants. We found an apparent rate constant for glibenclamide binding of 9.9×10^7 M^{-1} sec^{-1} and an unbinding rate of 6.26 sec^{-1}.

(4) Glibenclamide is a lipophilic molecule and is likely to act on sulfonylurea receptors from within the hydrophobic phase of the cell membrane. The glibenclamide concentration within this phase will be greater than that in the aqueous solution and we have taken this into account to estimate a true binding rate constant of 1.66×10^6 M^{-1} sec^{-1}.

Key words: K_{ATP} — ATP — Glibenclamide — ATP — Kinetics

Introduction

Potassium channels closed by internal ATP (K_{ATP} channels) are widely distributed in mammalian tissues (reviewed by Ashcroft & Ashcroft, 1990). They were first identified by Noma (1983) in cardiac muscle cells where they are believed to couple metabolic state to membrane excitability. Subsequently, K_{ATP} channels have been identified in pancreatic B-cells, where they link insulin release to blood glucose levels, and in skeletal muscle where their function is less clear (reviewed by Davies, Standen & Stanfield, 1991). Inagaki et al. (1995, 1996) proposed recently that the K_{ATP} channel is actually a complex of a weak inward rectifier channel (Kir6.2) coupled closely in the membrane to a receptor for sulfonylureas (SUR). In this model, the Kir6.2 channel protein confers the properties of conduction and ion selectivity, whereas the SUR protein serves as the receptor for ATP and pharmacological modulators of channel function such as glibenclamide and pinacidil. Glibenclamide, a sulfonylurea, is a very potent inhibitor of K_{ATP} channels in cardiac and pancreatic cells having K_i values of 2 and 0.2 nM respectively (Schmid-Antomarchi et al. 1987*a*,*b* and Fosset et al. 1988). Glibenclamide is, however, much less active on K_{ATP} channels of skeletal muscle and has been proposed to act at a functionally distinct subtype of SUR (SUR2, Inagaki et al., 1996). Standen et al. (1992), found that application of glibenclamide to amphibian sartorius muscle inhibited K_{ATP} channels with a K_i of 3μM. More recently, using outside-out patches of mouse skeletal muscle, Allard and Lazdunski (1993) obtained a K_i for glibenclamide of 190 nM, about 1,000-fold greater than that measured in pancreatic B-cells.

We have investigated the action of glibenclamide on rat skeletal muscle K_{ATP} channels in order to determine the K_i, the binding stoichiometry and make estimates of the rate constants for the binding and unbinding of glibenclamide to the K_{ATP} channel complex.

Materials and Methods

Preparation

Single muscle fibers were isolated from rat flexor digitorum brevis muscle (F.D.B.) using collagenase as described previously (McKillen et al. 1994).

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Solutions

The extracellular solution, used to fill the patch pipette, had the following composition (mM): KCl, 10; NaCl, 145, $CaCl_2$, 2; HEPES, 10, pH was adjusted to 7.4 with NaOH. The internal solution contained (mM): KCl, 99; EGTA, 5; K-fluoride, 40; HEPES, 10, pH was adjusted to 7.4 with KOH. K-Fluoride was included in the solution to reduce channel 'rundown' (McKillen et al. 1994). $MgCl_2$ 1.4 was included in the internal solution only where stated. Glibenclamide was purchased from Sigma and dissolved in DMSO to make a 100 mM stock solution. The highest concentration of glibenclamide used was 10 μM, thus the maximum DMSO exposure was 0.01%. DMSO applied to membrane patches at 0.1% was found to be without effect.

Recording Methods

K_{ATP} channel currents were recorded using excised, inside-out membrane patches (Hamill et al., 1981). Patch pipettes were pulled from thick-walled borosilicate tubing, coated with Sylgard resin, and fire polished. Pipette resistance was 10–20 MΩ when filled with pipette solution. Following seal formation, patches were excised and placed in a switchable stream of intracellular solution. Currents were measured with a List EPC-7 amplifier filtered at 10kHz and stored on videotape at 40kHz for off-line analysis.

Analysis

Prerecorded data were replayed through an 8-pole Bessel filter at a cutoff frequency (-3dB) of 4kHz and digitized at 20kHz using a TL-1 A-D interface (Axon Instruments, Foster City, CA). A 50% threshold was used to detect open and closed events, linear regressions joining pairs of data points on either side of the threshold giving the estimated times of crossing. A minimum resolution (t_{min}) of 100μsec was imposed on the data and the remaining events were log-binned at 25 bins per $\log_{10}$ unit according to the method of Sigworth and Sine (1987). Distributions were fitted to binned data by the method of maximum likelihood to the probability density function:

$$f(t) = \sum_{j=1}^{m} (a_j/\tau_j)\exp(-t/\tau_j) \quad (1)$$

where α_j is the area, τ_j is the time constant of component j, and m is the number of components. In general, openings were interrupted by brief closings, many of which were undetected, resulting in an overestimation of the measured mean open time, t_o. This was corrected by multiplying it by the proportion of closed events detected (given by the integral of the fitted closed time distribution between t_{min} and infinity).

Bursts were defined as an opening or series of openings separated by closures shorter than a critical time t_c. This time was calculated from the closed time distribution by assigning the three shortest components as gaps within bursts and equalizing the proportion of short closings, misclassified as long, and of long closing, misclassified as short (Colquhoun and Sakmann, 1985).

Channel activity was calculated as NP_{open}, which is given by $\Sigma t_j J/T$, where t_j is the time spent at level j ($1 \ldots N$), T is the total duration of the recording, N is the number of channels and P_{open} is the open probability. Fractional block is then NP_{open} in the presence of glibenclamide divided by that before. For patches with a large number of channels where P_{open} could not be calculated, the average current was measured instead.

Dose responses were fit with the equation:

$$F = 1 - \frac{c^h}{c^h + K_i^h} \quad (2)$$

where F is the fractional block, K_i is the concentration giving half maximal block, and h is the Hill coefficient.

All experiments were carried out at room temperature, 20–25°C, and results are given as means ± their standard errors, or the asymptotic standard errors returned by the curve fitting routines of Sigmaplot 5.0 (Janel Scientific).

Results

Holding excised inside-out patches of F.D.B. muscle at 0 mV we saw channel currents with unitary amplitudes of approximately 2pA. These were confirmed as K_{ATP} channels by a reduction of their activity by application of cytosolic ATP (McKillen et al., 1994). The number of channels in any one patch was variable. For the construction of the dose response curve we selected patches with several channels, and for kinetic analysis we looked at patches with as few active channels as possible, limiting our measurements to periods when only one channel was active. In patches containing more than one active channel it is not possible to obtain the closed time distribution, but the mean closed time, t_c, could be estimated as $t_o(1 - P_{open})/P_{open}$ where t_o is the corrected mean open time (*see above*).

Inhibition of K_{ATP} Channels by Glibenclamide

Application of low concentrations of glibenclamide to the internal face of the membrane rapidly and reversibly inhibited K_{ATP} channels, Fig. 1*A*. At higher concentrations, reversal of the glibenclamide inhibition was very slow (*data not shown*), and may reflect the high solubility of this compound in the membrane (*see discussion*). Figure 1*B* shows the dose-response relation for the inhibition of channel activity by glibenclamide in the absence of Mg^{2+}. The solid line shows the best fit of Eq. (2) to the data which gave a K_i of 63 ± 17 nM and a Hill coefficient, h, of 0.85 ($n = 16$ patches). We found that internal Mg^{2+} (1.4 mM) did not affect significantly the sensitivity of the skeletal muscle K_{ATP} channel to glibenclamide (Fig. 1; $K_i = 78 \pm 52$ nM, $h = 0.9 \pm 0.5$, $n = 4$ patches).

Kinetic Analysis of Glibenclamide Inhibited K_{ATP} Channels

In patches containing only one or two active K_{ATP} channels it was clear that openings occurred in bursts separated by closed times that were occasionally long lasting. This is typical of the behavior of these channels in other preparations (Spruce et al. 1987; Davies, Standen & Stanfield 1992; McKillen et al. 1994). Figure 2 shows recordings of a single K_{ATP} channel in the absence and presence of 1 μM glibenclamide. The most noticeable effect was a reduction in burst duration and an increase in closed time duration. This is shown quantitatively in Fig. 3 by the distributions of open time, closed time and

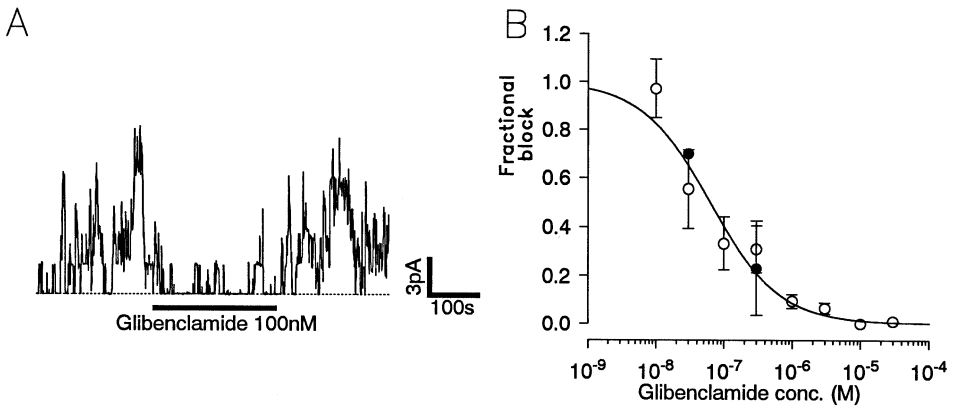

Fig. 1. Glibenclamide inhibits K_{ATP} channels rapidly. (*A*) Application of 100 nM glibenclamide to the internal face of the membrane (in the absence of Mg^{2+}), holding potential 0 mV. (*B*) Dose-response curve for the action of glibenclamide on 16 inside-out patches of skeletal muscle membrane: (○) zero magnesium, (●) 1.4 mM Mg^{2+}. The data (in the absence of magnesium) were fit with Eq. 2. Half maximal block, K_i was 62.9 nM and the Hill coefficient was 0.85.

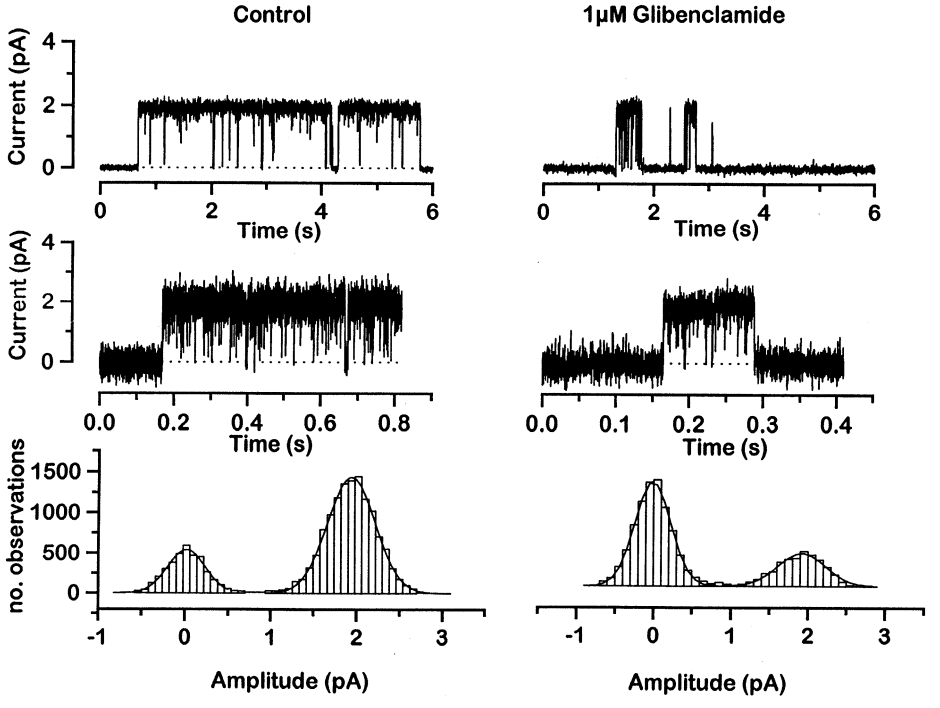

Fig. 2. Glibenclamide reduces burst lengths. The panels on the left show control data, panels on the right are in the presence of 1 μM internal glibenclamide. The top row show data at low time resolution (sample interval 750 μsec, filtered at 1KHz). The middle row show sections of the above data, but at our maximum resolution (sample interval 50 μsec, filtered at 4KHz). Burst lengths are shorter in the presence of glibenclamide, but there is less obvious change of fast events. The bottom two panels show all points amplitude histogram plots from the data above. The histogram on the left (in the absence of glibenclamide) is fitted with Gaussians of 0.02 ± 0.22 and 1.94 ± 0.28. The histogram on the right is fitted with Gaussians of −0.01 ± 0.47 and 1.92 ± 0.31.

burst duration in the presence and absence of glibenclamide. Control dwell times were well fitted assuming three open states (corrected mean open time, *cmot* = 6.27 ± 0.38 msec, $n = 5$ patches) and five closed states (mean closed time, $t_c = 6.20 \pm 0.38$ msec, $n = 5$ patches). The mean burst duration in these patches was 411 ± 19 msec. This is somewhat longer than reported previously in amphibian muscle (Spruce et al. 1987, Davies et al. 1992).

In the presence of 1 μM glibenclamide, corrected mean open times were reduced by 55% (*cmot* = 2.8 ± 0.1 msec, $n = 3$ patches), while mean burst lengths were

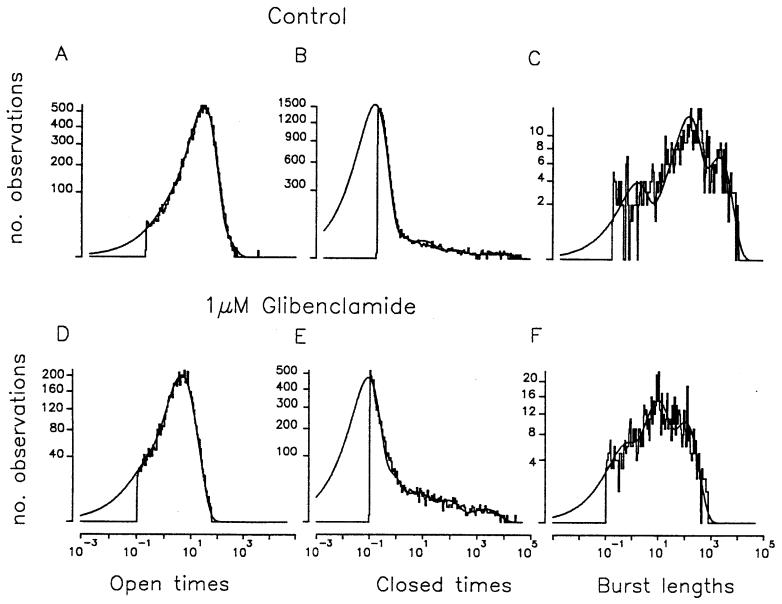

Fig. 3. K_{ATP} channel kinetics. Distribution of open times, closed times and burst lengths in control (*A, B,* and *C*), and in the presence of 1 μM glibenclamide (*D, E,* and *F*). Eq. (1) was fitted to these distributions with 3 components for the open times, 5 for the closed times and 3 for burst lengths. The values returned for the fits were (area:time constant, msec): Control open times; 0.03:0.57, 0.94:13.76, 0.03:48.30. Control closed times; 0.97:0.09, 0.02:0.5, 0.01:5.3, 0.003:78.5, 0.001: 3065. Control burst lengths; 0.15:0.8, 0.54:73.99, 0.31:1070. 1 μM glibenclamide open times; 0.085:0.48, 0.58:7.43, 0.33:15.47. 1 μM glibenclamide closed times: 0.645:0.05, 0.31:0.14, 0.024:2.5, 0.015:78.2, 0.007:1960. 1 μM glibenclamide burst lengths; 0.4:0.8, 0.3:25.98, 0.28:254.

reduced by 75% of the control level (89.0 ± 9.9 msec, $n = 3$ patches). The larges change, however, was an 18-fold increase in mean closed time (61.3 ± 1.1 msec, $n = 3$ patches).

Since open times are reduced in a concentration-dependent manner by glibenclamide, and since high concentrations of glibenclamide lead to complete block, it is reasonable to assume that binding of glibenclamide leads to channel closure. For a system with k open states the general solution for the reciprocal of the mean open time is given by:

$$\frac{1}{\overline{t_o}} = \sum \frac{P_i}{P_o} h_i \quad i = 1 \ldots k \tag{3}$$

where P_i is the steady state occupancy of the ith open state, P_o the open probability and h_i the sum of the rate constants leading from the ith open state to any shut state (Colquhoun & Hawkes, 1977; Davies *et al.*, 1989). In the presence of a blocker, B, Eq (3) can be written conveniently as:

$$\frac{1}{\overline{t_o}} = \sum \frac{P_i}{P_o} h_i + \left(\sum \frac{P_i}{P_o} b_i \right) [B] \tag{4}$$

where b_i represents the sum of rate constants leading from the ith open state to any blocked state. The value within the brackets in Eq. (4) is the weighted mean of the rate constants for blocking each of the open states. If these rates are the same for each open state, then this bracketed term simplifies to k_b, the glibenclamide binding rate.

An estimate of the binding rate can thus be obtained as the slope of a plot of glibenclamide concentration against the reciprocal of the mean open time (Fig. 4*A*). The slope of the line in Fig. 4*A* gave a binding rate of 9.9×10^7 M^{-1} sec^{-1}. With a Hill coefficient near unity, the dissociation constant K_D is equal to K_i (*see* Eq. 2), and the unbinding rate could be calculated as $k_{-b} = K_D \cdot k_b$, which gave $k_{-b} = 6.26$ sec^{-1}.

Glibenclamide is a rather lipophilic molecule and in cardiac muscle it has been suggested to bind to its receptor from within the cell membrane (Findlay, 1992). In this case our calculated blocking rate constant would be too high. Findlay (1992) estimates that at pH 7.4 the concentration of glibenclamide within the membrane will be about 60 times higher than in the aqueous solution (glibenclamide has a pK_a of 6.3 and an octanol:water partition coefficient of 1200). If glibenclamide acts from within the membrane then our calculated binding rate should be modified to 1.66×10^6 M^{-1} sec^{-1}. Our estimated unbinding rate of 6.26 sec^{-1} will be unaffected by this.

Figure 4*B* shows mean burst lengths, open times and closed times plotted against glibenclamide concentration. At 100 nM, nearly double the K_i, burst lengths and open times were reduced by only 25% and 18% respectively, whereas mean closed time was increased more than threefold. These results are broadly similar to those found by Gillis et al. (1989) who examined the kinetic action of sulfonylureas in pancreatic B-cells and found that 10 μM tolbutamide reduced mean burst lengths by 66%, and mean open times by less than 20%.

Discussion

We have shown here that glibenclamide can inhibit K_{ATP} channel activity with a stoichiometry of 1:1 and a K_i of

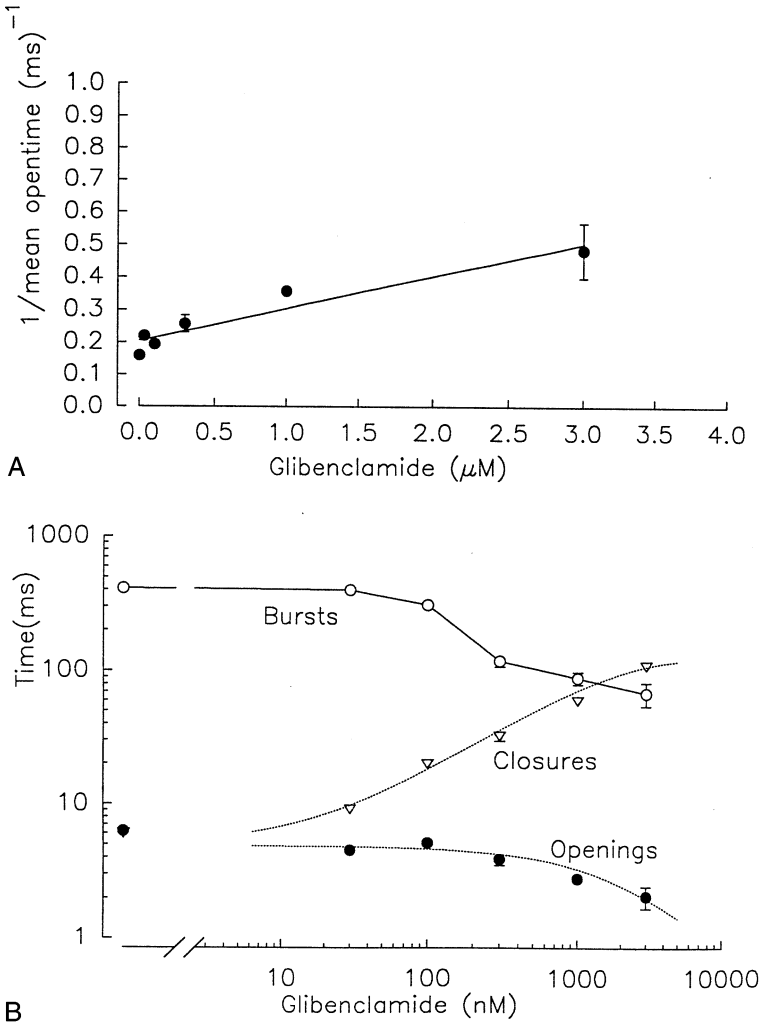

Fig. 4. Reduction of mean open time by glibenclamide. (*A*) Reciprocal corrected mean opentime against glibenclamide concentration. The line is the fit to:

$$1/t_o = k_b.[Glib] + 1/t_{o([\mathrm{Glib}] = 0)} \quad (5)$$

where k_b is the binding rate constant, t_o is the *cmot* in the presence of glibenclamide and $t_{o([\mathrm{Glib}] = 0)}$ is the *cmot* with no blocker. (Data from 6 patches). (*B*) Mean dwell times plotted against the concentration of glibenclamide. Bursts (open circles), corrected openings (closed circles) and closures (triangles). The broken lines through the openings and closures are drawn from the calculated rate constants for binding and unbinding of glibenclamide. Note that both abscissa and ordinate are log scales (Data from 6 patches).

63 nM when added to the cytosolic side of inside-out patches of rat skeletal muscle membrane. This K_i is similar to that of 190 nM measured in mouse skeletal muscle by Allard and Lazdunski (1993), but is much lower than that observed in amphibian skeletal muscle (3 μM, Standen et al., 1992). The recent work of Inagaki et al. (1995, 1996) shows that the K_{ATP} channel exists as a complex of a Kir 6.2 channel and an SUR protein. Furthermore, the SUR receptor found in cardiac and skeletal muscle (designated SUR2) is less sensitive to both ATP and sulphonylureas than that found in pancreatic B-cells. Our value of 63 nM for the K_i in skeletal muscle, being approximately 300-fold higher than that found in pancreatic cells (Schmid-Antomarchi et al, 1987), is consistent with this finding.

K_{ATP} channel kinetics have been studied previously in skeletal muscle (Davies et al. 1989, 1992) and in pancreatic B-cells (Gillis et al. 1989). In both tissues, the kinetics were shown to be complex, having several open and closed states. Gillis et al. (1989) found that both sulfonylureas and glucose (acting through ATP) decreased burst duration and increased closed times. In this study we have shown that glibenclamide decreases mean open time in a concentration-dependent manner, suggesting that glibenclamide binding leads to channel closure. In fact, since it is the SUR protein, and not the channel itself, which serves as the target for sulfonylureas, it is likely that the binding of glibenclamide does not depend on whether the channel is open or closed. Despite the complex kinetics of these channels it was still possible to extract valuable information about blocking and unblocking rate constants from the relation between mean open time and glibenclamide concentration. Our analysis, after accounting for the partition coefficient of glibenclamide in the membrane (Findlay, 1992), gave blocking and unblocking rate constants of 1.66×10^6 M^{-1} sec^{-1} and 6.26 sec^{-1} respectively.

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