Activator-induced dynamic disorder and molecular memory in human two-pore domain hTREK1 K⁺ channel

Tapan Kumar Nayak · Saswati Dana ·
Soumyendu Raha · Sujit K. Sikdar

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Abstract Ion channels are fundamental molecules in the nervous system that catalyze the flux of ions across the cell membrane. Ion channel flux activity is comparable to the catalytic activity of enzyme molecules. Saturating concentrations of substrate induce “dynamic disorder” in the kinetic rate processes of single-enzyme molecules and consequently, develop correlative “memory” of the previous history of activities. Similarly, binding of ions as substrate alone or in presence of agonists affects the catalytic turnover of single-ion channels. Here, we investigated the possible existence of dynamic disorder and molecular memory in the single human-TREK1-channel due to binding of substrate/agonist using the excised inside-out patch-clamp technique. Our results suggest that the single-hTREK1-channel behaves as a typical Michaelis–Menten enzyme molecule with a high-affinity binding site for K⁺ ion as substrate. But, in contrast to enzyme, dynamic disorder in single-hTREK1-channel was not induced by substrate K⁺ binding, but required allosteric modification of the channel molecule by the agonist, trichloroethanol. In addition, interaction of

trichloroethanol with hTREK1 induced strong correlation in the waiting time and flux intensity, exemplified by distinct mode-switching between high and low flux activities. This suggested the induction of molecular memory in the channel molecule by the agonist, which persisted for several decades in time. Our mathematical modeling studies identified the kinetic rate processes associated with dynamic disorder. It further revealed the presence of multiple populations of distinct conformations that contributed to the “heterogeneity” and consequently, to the molecular memory phenomenon that we observed.

Keywords TREK1 · Ion channel · Dynamic disorder · Molecular memory · Ion flux

Introduction

Ion channels perform the important task of ion transport across the membrane along the electro-chemical gradient. The popular constant field GHK equation [13] predicts that ion channel conductance, i.e., the rate of ionic flux through the pore of an open channel should increase with increasing ion concentration. However, experimental observations in different ion channels revealed that single-ion channel conductance saturates with increase in ion concentration [15, 17, 29]. The hyperbolic dependence of channel conductance on the concentration of permeable ions is similar to the saturation kinetics observed in the case of enzymes and has been described by the classical Michaelis–Menten (MM) equation [16]. The remarkable similarity led to the idea that ion channels operate as enzymes catalyzing the flux of ions, with the

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T. K. Nayak · S. K. Sikdar (✉)
Molecular Biophysics Unit, Indian Institute of Science,
Bangalore 560012, India
e-mail: sks@mbu.iisc.ernet.in

S. Dana · S. Raha (✉)
Supercomputer Education and Research Centre,
Indian Institute of Science,
Bangalore 560012, India
e-mail: raha@serc.iisc.ernet.in

involvement of an ion binding step, akin to the enzyme substrate reaction. Furthermore, X-ray crystallographic studies on various ion channels have confirmed that ionic flux involves binding of ions to a small number of definite, high-affinity binding sites along the channel pore [7, 32].

Recent studies on single-enzyme molecules using high-resolution spectroscopic tools suggest that their behavior is stochastic in real time, analogous to ion channels [30]. At high substrate concentration, a “molecular memory” phenomenon arises in single-enzyme molecules, characterized by highly correlated turnover events, which persists for decades of timescale ranging from milliseconds to seconds [10, 11]. Molecular memory in a single-enzyme molecule is attributed to “dynamic disorder” induced by substrate binding. It refers to fluctuations in kinetic rate processes, broad distribution of catalytic rate constant k_{cat} and is associated with slow conformational dynamics in the timescale of 10^{-4} – 10 s [10, 25]. On the other hand, single-ion channels exhibit fluctuations in their activity owing to conformational changes due to permeant ion binding, which have been captured by single-channel patch-clamp recordings [6]. Ion channels also have specific agonists which upon binding induce large changes in the conductance and kinetic properties of the channel [6]. However, the possibility of substrate/agonist-induced dynamic disorder and molecular memory has not been explored in ion channels. In the present work, we studied this aspect of the single human-TREK1-channel using the excised inside-out patch-clamp recording technique in the absence or presence of the agonist trichloroethanol (TCE), the active metabolite of the general anesthetic and sedative, chloral hydrate.

TREK1, a member of the two-pore domain K^+ channels, is known to be activated by a wide array of extracellular ligands and pharmacological reagents, including TCE [14]. However, it lacks an elaborate gating mechanism and exhibits a basal level activity in absence of agonists, unlike the ligand-gated ion channels [18]. This enables the measurement of induced activity over the basal level, thus making it an ideal system to study conformational dynamics in absence or presence of agonists. The catalytic turnover of the hTREK1 channel was studied by transforming the prototypic single-channel activity into ionic flux values. The possible induction of dynamic disorder and molecular memory was probed by studying the waiting-time distribution, two-dimensional joint probability distribution and autocorrelation analysis of successive waiting times and flux intensities. The saturation and anomalous mole fraction behavior of hTREK1 was studied to draw a comparison between the ion channel and single-enzyme molecule. Our results

suggest that the single hTREK1 molecule behaves similar to a single Michaelis–Menten enzyme molecule. However, an interesting deviation was observed in case of the hTREK1 channel. While dynamic disorder in the enzyme molecule was induced by substrate binding, in case of hTREK1, the substrate ion itself did not induce dynamic disorder, but required strong allosteric interaction of the agonist, TCE with the channel molecule. Concomitant with this, a strong correlation in the waiting time and flux intensity was observed, exemplified by distinct mode-switching between long stretches of high and low flux activities. This illustrated the induction of molecular memory in the channel molecule by the agonist, which persisted for several decades in time. Moreover, our mathematical modeling studies identified the rate processes with dynamic disorder and explained the induction and persistence of the memory phenomenon in the single hTREK1 channel molecule.

Materials and methods

Maintenance of HEK 293 (Human Embryonic Kidney) cells, generation and delivery of recombinant hTREK1 adenovirus (Ad-hTREK1) and assay of hTREK1 expression in Ad-hTREK1-infected HEK 293 cells has been described elsewhere ([34]). The single-channel current recordings were carried out in HEK 293 cells infected with Ad-hTREK1 in the excised inside-out configuration of patch-clamp technique. Statistical analysis of single-channel data was done offline on the recorded data by the Pulse (HEKA Elektronik, Germany) and TAC software (Bruxton Corp, Seattle, Washington). Two-dimensional joint probability distribution and autocorrelation analyses were performed as described earlier [10, 30]. Most of the experiments were done in symmetrical gradient of potassium ions, where the pipette and bath solutions contained: 155 mM KCl, 5 mM EGTA, 3 mM $MgCl_2$, and 10 mM HEPES (pH adjusted to 7.4 with KOH). For the study of selective permeability of hTREK1, 1 M stock of the cationic solutions (Li^+ , Na^+ , Cs^+ , Rb^+ , and NH_4^+) were prepared from their respective chloride salts and the experimental bath and pipette solutions were prepared by replacing KCl with equimolar concentration of cations. The resulting solutions were adjusted for pH by the hydroxides of their respective cation. In experiments to study the saturation behavior of hTREK1, K^+ was replaced with choline whereas in anomalous mole fraction behavior experiments, K^+ was replaced by increasing concentrations of NH_4^+ or Cs^+ . In all conditions, the osmolarity and the pH of the solution were maintained at 300 mOsm and 7.4, respectively. Test solutions of 2,2,2-trichloroethanol (TCE, 10 mM stock; Aldrich) was freshly

prepared in the extracellular saline solution and applied very close to the patch by a hydrostatic pressure driven fast perfusion device (VC-6; Warner Instruments Corp., CT), while continuously perfusing the bath with extracellular solution. All the single-channel current recordings in presence of TCE were done at room temperature and only in the steady state at a constant membrane voltage of -60 mV. Results have been presented as the mean $\pm$ standard error and the significance at 95% confidence level was verified by the Student's t test or non-parametric test such as the Mann–Whitney U test.

Conversion of single-channel activity into flux

The single-channel activity of hTREK1 was converted into flux activity by integrating the area under the single-channel current-time trace. This was accomplished by estimating the product of the single-channel current (i) and the sampling interval ($50 \mu\text{s}$, Δt) at every sample point and adding to the previous product if the sample resided in the same conductance level as the previous one as follows:

$$Q = \sum_{i=1}^n i \cdot \Delta t \quad (1)$$

The distinction between an individual open event and a burst was done by using a “burst-merge” criterion which was user defined. If two independent events appeared in the limit of burst-merge, they were summed and represented as a single flux activity. The burst-merge criterion was determined by estimating the intra-burst close time (by plotting close time histograms and fitting with the probability density function (pdf)) and it varied depending on the recording conditions. The stringency of the analysis was determined by the choice of the burst-merge criterion. The flux activity was represented at the midpoint of the open event or the burst. The duration between successive flux activities was defined as the “waiting time” for product formation. Each flux activity represented a complete catalytic cycle and provided estimate of the turnover rate of the channel.

Theoretical modeling

The ordinary differential equations (ODE) model (Eqs. 2 to 6) contains the chemical reaction rate constants as parameters which are not static constants due to the nature of the ion channel– K^+ interaction. The ion channel is a large molecule in dynamic equilibrium with K^+ rather than being statically stable. The vibrating molecule changes the reaction rate constants according to its modal shape. Hence seemingly a wide range of models with various rate constants may fit the data. It is not possible to observe all the modal shapes, but

the experiment ends up recording or observing principal modes. The effective model is thus the one which is a weighted average of the principal modes of the ion channel– K^+ ion binding. But these weights as well as the principal reaction constants have to be estimated from the experimental data. This estimation is done by the optimization process (Eqs. 11 and 12). It preserves the data fidelity of the model to the experiment by not force fitting any particular model onto the data. Hence the estimates about the principal reaction modes and their weights come entirely from the experimental data. Thus, the dynamic disorder computation and verification that follow the estimates are due to the experimental data only and not based on any assumption on the modes of the reactions.

The channel dynamics in presence of K^+ ions alone could be explained by solving a set of three ODEs as in the case of single-enzyme molecule [25]. In the presence of the agonist, TCE, the dynamics of the system could be written as a set of five linear ODEs as follows:

$$\frac{dP_C(t)}{dt} = -(k_1 + k_a)^0 P_C(t) + k_{-1} P_{CS_i}(t) + k_{-a} P_{CA}(t) \quad (2)$$

$$\frac{dP_{CS_i}(t)}{dt} = k_1^0 P_C(t) - (k_2 + k_{-1} + k_a) P_{CS_i}(t) + k_{-a} P_{CAS_i}(t) \quad (3)$$

$$\frac{dP_{CA}(t)}{dt} = k_a P_C(t) + k_{-1} P_{CAS_i}(t) - (k_a + k_1')^0 P_{CA}(t) \quad (4)$$

$$\frac{dP_{CAS_i}(t)}{dt} = k_a P_{CS_i}(t) - (\beta k_2 + k_{-1}' + k_a) P_{CAS_i}(t) + k_1'^0 P_{CA}(t) \quad (5)$$

$$\frac{dP_{C^0}(t)}{dt} = k_2 P_{CS_i}(t) + \beta k_2 P_{CAS_i}(t) \quad (6)$$

This system of ODEs can be rewritten in the form of

$$\dot{P} = Ap \quad (7)$$

where $P(0) = P_0$

It has a unique solution for all real t and is given by

$$p(t) = e^{At} P_0 \quad (8)$$

Here, A is a 5×5 matrix of kinetic rate constants given by:

$$A = \begin{pmatrix} -(k_1 + k_a)^0 & k_{-1} & k_{-a} & 0 & 0 \\ k_1^0 & -(k_2 + k_1 + k_a) & 0 & k_{-a} & 0 \\ k_a & 0 & -(k_1' + k_a)^0 & k_{-1} & 0 \\ 0 & k_a & k_1'^0 & -(\beta k_2 + k_{-1}' + k_a) & 0 \\ 0 & k_2 & 0 & \beta k_2 & 0 \end{pmatrix}$$

Since the solution involves the exponentiation of the matrix, it is difficult to solve analytically. Therefore, we resorted to a numerical method for solving the equations. The *ode15s* of MATLAB was utilized to obtain the numerical solution. Under steady state in the presence of TCE, the pdf of the waiting times was given by

$$f(t) = \frac{dP_{C^0}(t)}{dt} = k_2 P_{CS_i}(t) + \beta k_2 P_{CAS_i}(t) \quad (9)$$

where P_{CS_i} and P_{CAS_i} were obtained from the numerical solution. In presence of TCE, the induction of dynamic disorder was modeled assuming at least three slowly interconverting conformers with different k_2 , such that $f(t)$ was the weighted average of $f_{T_{C_1}}(t)$, $f_{T_{C_2}}(t)$, and $f_{T_{C_3}}(t)$ as follows:

$$f(t) = \sum_{i=1}^3 w_i f_{T_{C_i}}(t) \quad (10)$$

where $f_{T_{C_i}}$ is the distribution of the waiting time T_{C_i} for the channel and $\sum_{i=1}^3 w_i = 1$, where w_i are the corresponding weights of the conformers. The optimization problem is then formulated as follows:

$$\min_{w_{j,q}} J_p = \sum_{i=1}^M \left\| f(p_i; q_j; t_i) w_j - \hat{f}_l \right\|_2^2, t_i \in [T_0, T_f] \quad (11)$$

Such that

$$\sum_{j=1}^N w_j = 1; w_j > 0 \quad (12)$$

$$q := (k_1, k_{-1}, k_1', k_{-1}', k_a, k_{-a}, \alpha, \beta, k_2^{\text{conformer1}}, k_2^{\text{conformer2}}, k_2^{\text{conformer3}})^T > 0$$

where $f(p_i; q; t_i)$ and $\hat{f}_l$ are the theoretical and experimental waiting-time distributions respectively at time point t_i . The equations were solved by a standard sequential quadratic programming (SQP). The routine *fmincon* in MATLAB provided the necessary SQP functionality. This kind of error minimization with probability measure as an optimization variable has been used to obtain kinetic parameters [2] and study uncertainty in experimental setups [1].

Results and discussion

The function of ion channels is usually studied by analyzing the current waveform from an ensemble. Single-ion channel proteins exhibit a great deal of conformational heterogeneity in their structure [12, 23, 38], which generally gets averaged out in a population. Such changes in conformation may manifest as heterogeneity in kinetic rate processes of ion channel turnover that may be attributed to “dynamic disorder,” analogous to single-enzyme molecule studies [8, 10, 30, 48]. Therefore, in the current work, we have explored the possible existence of dynamic disorder and its role in modulating the single-ion channel turnover using the hTREK1 channel as a model system. HEK 293 cells infected with Ad-hTREK1 exhibited high expression levels of the channel as compared with the uninfected and Ad-GFP-infected cells. The hTREK1 channel, when expressed in HEK 293 cells, formed functional channels with characteristics similar to that described earlier [34]. The single-channel slope conductance measured from the current–voltage relationship was found to be 95.7 ± 9.2 pS ($n=14$), which was in agreement with earlier observations [3, 18].

Ion selectivity of the hTREK1 pore

In physiological potassium gradient, single-channel hTREK1 current in inside–out patches reversed at close to -90 mV (Fig. S1a in the Electronic supplementary material), whereas in symmetrical K^+ gradient the reversal potential shifted to 0 mV (Fig. S1b in the Electronic supplementary material), which are characteristics of a channel highly selective for K^+ . In order to study the relative permeability sequence of hTREK1, we performed bi-ionic, single-channel reversal potential measurements in excised patches. The reversal potentials revealed an Eisenmann type IV relative permeability series ($K^+ > Rb^+ > NH_4^+ > Cs^+ > Na^+, Li^+$) as observed for voltage-gated and inwardly rectifying K^+ channels (Fig. S1c and Table 1 in the Electronic supplementary material). Like other K^+ channels, hTREK1 also exhibits tight regulation over the conductance of monovalent cations, where K^+ was readily conducted, as observed in single-

channel recordings by application of membrane voltage ramps (Fig. S1c versus Fig. S1b in the Electronic supplementary material).

Saturation kinetics and mole fraction dependence of single-hTREK1-channel conductance

The dependence of single-channel conductance on ionic concentration was measured in single-channel excised inside-out patches in symmetrical K^+ gradient as illustrated in Fig. 1a. With increase in K^+ ion concentration, the frequency of openings and current amplitude of single hTREK1 channel progressively increased (Fig. 1a). The slope conductance of hTREK1 channel at different concentrations of K^+ was measured from the linear part of the single-channel current–voltage relationship as illustrated in Fig. 1b. With increasing K^+ , the slope conductance of the channel reached a plateau beyond 100 mM K^+ (Fig. 1c). The concentration-dependence of conductance was fitted

with the single-channel Michaelis–Menten equation as follows:

$$g = \frac{g_{\max}[S_i]}{(K_m + [S_i])} \quad (13)$$

where, g is the single-channel conductance, S_i , the symmetrical ion concentration, and K_m is the Michaelis–Menten constant. The maximum conductance for the hTREK1 channel was found to be 101.8 ± 11.7 pS ($n=5-7$), whereas the K_m was estimated to be 26.4 ± 5.8 mM ($n=5-7$, Fig. 2c). The K_m is smaller than that observed for the related K_{2P} channel KCNKØ [21]. Behavior of this type suggests the presence of saturable binding site(s) within the channel and is typically found in channels that can be occupied by a single ion at a time [27, 29].

This was further validated by studying the mole fraction dependence of single-channel conductance. One of the most reliable tests for multi-ion occupancy is the occurrence of

Fig. 1 $[K^+]$ dependent single hTREK1 channel conductance.

a 10-s long traces of single-channel activity recorded in presence of increasing concentrations of K^+ in symmetrical gradient at different membrane potentials (shown in the figure). Note the increase in single-channel current amplitude with K^+ concentration. The recording configuration is illustrated alongside the current traces. **b** Single-channel current–voltage relationship obtained at different concentrations of K^+ . The measured slope conductances (between 0 and 80 mV) were 31.2 ± 4.7 pS ($n=5$), 78.1 ± 8.5 pS ($n=5$), and 112.5 ± 7.4 pS ($n=7$), respectively in presence of 20, 50, and 150 mM K^+ . **c** Dependence of single-channel slope conductance on the concentration of K^+ . Note the saturation of conductance with increasing $[K^+]$. The solid curve is the model fit of the data with Michaelis–Menten equation ($g_{\max}$ and K_m are 101.8 ± 11.7 pS ($n=5-7$) and 26.4 ± 5.8 mM ($n=5-7$), respectively; data presented as mean $\pm$ SEM)

Fig. 2 Mole fraction dependence of unitary hTREK1 conductance. Single-hTREK1-channel current evoked by a ramp pulse in presence of bi-ionic salt solution of K^+ and NH_4^+ (a) and K^+ and Cs^+ (b) in symmetrical gradient. The mole fraction has been illustrated by the ratio of the salts in the figure. The recording configuration is illustrated alongside the current traces. Note the increase in ramp current with increase in K^+ mole fraction. Also note the complete block of single-channel current in presence of only Cs^+ ($K^+ : Cs^+ = 0 : 150$, uppermost panel (b) in a voltage-independent manner. **c** Dependence of conductance of hTREK1 on the mole fraction of K^+ in presence of NH_4^+ (filled triangles) and Cs^+ (empty squares), respectively. Note the monotonous rise in conductance with increasing mole fraction of K^+ in both cases

anomalous mole fraction behavior, where the single-channel conductance exhibits anomalous maximum and minimum values with increasing mole fraction [29]. For these experiments, the single-channel conductance was measured in a symmetrical gradient of mixed salt solution (K^+ and X^+) with a fixed total salt concentration, but with increasing mole fraction of K^+ , in the inside-out patch configuration (Fig. 2a, b). The results have been shown for mixed solutions of K^+ / NH_4^+ (Fig. 2a) and K^+ / Cs^+ (Fig. 2b) with the total salt concentration being 150 mM. The unitary conductance measured from such experiments was plotted against the mole fraction of K^+ (Fig. 2c). In both sets of experiments, the unitary conductance increased monotonically with K^+ mole fraction. This suggests the absence of mole fraction behavior in the hTREK1 channel. In essence, the hTREK1 channel exhibits attributes of a single-ion pore, similar to those described earlier for the sarcoplasmic reticulum K^+ channel, Kir 7.1 and KCNQ channels [20, 29, 39, 44]. Furthermore, we observed voltage-independent block of

hTREK1 current by Cs^+ (Fig. 2b, trace recorded in the presence of Cs^+ alone, $K^+ : Cs^+ = 0 : 150$). It suggests that Cs^+ blocks the pore only superficially, outside the electrical field of the pore in the channel [44] which provides additional evidence in favor of a single-ion pore. The results above suggest that the hTREK1 channel exhibits substrate (K^+)-dependent saturation kinetics analogous to the Michaelis–Menten enzymes and behaves as a single-ion pore, most likely, with a single high-affinity binding site for the substrate ion.

Description of hTREK1 channel as a single-enzyme molecule

The presence of a distinct ion binding site in the channel molecule is the basis for the chemical-kinetic description of the channel mediated ionic flux [32]. Essentially, the binding of an ion to the channel is analogous to the binding of the substrate to an enzyme molecule. Therefore, the

ensemble behavior of such a system can be described [16, 28, 36] as:

where, S_i , S_0 are the internal, external ions [K^+ in this case] and the CS_i is the intermediate complex of channel, C and K^+ ion. However, unlike the ensemble, the single-channel behavior is stochastic. Recordings from single-channel molecules yield measurable quantities such as the close–open dwell times and the corresponding conductances. The probability density function obtained by recording the successive dwell times provides an estimate of the mean dwell time of the channel. But the classical single-channel events may or may not represent the catalytic cycle given in Eq. 14. An independent open event represents the channel opening and subsequent closure (complete catalytic cycle), whereas a burst activity is a composite of close–open events where the channel might remain in conducting state throughout the burst duration. Hence, the intra-burst open channel events may not represent a catalytic cycle but a part of it. Therefore, the single-channel activity from hTREK1 was converted into ionic flux activity by integrating the area under the recorded traces using the burst-merge criteria, as described in the methods. Figure 3 illustrates the single-channel flux activity, which is stochastic in nature and each single-channel flux activity represented a complete catalytic cycle. The time between two successive flux activities represents the waiting time for the next catalytic cycle or product formation. The probability density function of the waiting times, $f(t)$ provided information about the mean waiting time and the turnover rate of an ion channel. This is analogous to the single-enzyme molecule studies where the turnover rates were

Fig. 3 Transformation of single-channel activity into ionic flux activity. The *upper panel* shows typical single hTREK1 channel activity with random open, close events and bursts of activity. The *lower panel* is the flux activity obtained by integrating the area under the single-channel activity trace (see “Materials and methods”). *c.u.*: charge unit representing the time integration of single-channel current

estimated by measuring the waiting times between successive “on” states, recorded as fluorescence intensities from the burst of fluorescent product molecules per unit time [10, 30].

Theoretical interpretation of the single hTREK1 channel flux activity

In a single-channel turnover/flux measurement experiment as described above, the channel molecule is monitored continuously as it cycles repeatedly through C , CS_i , and C^0 . The time for the first reaction to complete is a stochastic variable and has been described by the pdf of the waiting times $f(t)$. Under steady-state assumption the pdf was given by (see methods):

$$f(t) = \frac{dP_{C^0}(t)}{dt} = k_2 P_{CS_i}(t) \quad (15)$$

And

$$f(t) = \frac{k_1 k_2 S_i}{2A} [\exp(A + B)t - \exp(B - A)t] \quad (16)$$

where $A = \sqrt{(k_1[S_i] + k_{-1} + k_2)^2/4 - k_1 k_2 [S_i]}$, and $B = -(k_1[S_i] + k_{-1} + k_2)/2$. This analytical solution [10, 25] was used to describe the turnover of the ion channel in presence of different concentrations of K^+ .

Substrate K^+ ion binding does not induce dynamic disorder in the channel

In single-enzyme molecule studies, the presence of dynamic disorder was revealed by the analysis of the waiting times between successive on states. In order to study the waiting time distribution of the channel molecule upon binding of K^+ ion, we performed single-channel excised inside–out patch recordings of hTREK1 channel in symmetrical gradient of K^+ (Fig. 4a, c, and e). With increasing K^+ concentration, the channel conductance as well as the open probability progressively increased. The single-channel activity recorded at different $[K^+]$ was converted into ionic flux activity (see “Materials and methods”). The waiting time pdf was obtained by recording successive waiting times, plotting histograms and fitting them with exponential functions obtained from the solution in Eq. 15. At all concentrations of K^+ (20–150 mM) the waiting time distribution could be fitted with a mono-exponential function (Fig. 4b, d, and f). Each distribution had a distinct rising and falling phase, signifying the formation of a reaction intermediate during the catalytic cycle (Fig. 4b, d, and f). However, with increasing K^+ concentration the mean waiting time progressively reduced, highlighting the gradual increase in activity with increase in substrate concentration (Fig. 4b, d, and f). From

Fig. 4 Effect of increasing substrate K^+ concentration on the waiting-time distribution. **a**, **c**, and **e**—*i* Single-hTREK1-channel activity recorded in the inside-out configuration with progressively increasing concentration of $[K^+]$ (**a** 20, **c** 50, and **e** 100 mM, respectively). *ii* Ionic flux activity for the respective single-channel trace shown in (*i*). Note the increase in ionic flux with increasing K^+ concentration. Also

note that the flux activity is randomly distributed even at 100 mM K^+ . **b**, **d**, and **f** Waiting-time distribution histograms for the flux activity shown in (**a**, **c**, and **e**). The *solid curve* represents the probability density function (pdf) fitting the data. In each case, the pdf was a mono-exponential function. However, note the progressive decrease in the mean waiting time with increasing K^+ concentration

the theoretical modeling of the pdfs, the corresponding values of k_1 , k_{-1} , and k_2 were obtained. In presence of 150 mM K^+ the values are as follows: $k_1 = 1.25 \times 10^7 \text{ s}^{-1}$, $k_{-1} = 4,294 \text{ s}^{-1}$, and $k_2 = 10^6 \text{ s}^{-1}$. A kinetic rate process is said to have dynamic disorder when a discrete rate constant is replaced by a random function of time. This is often marked by a deviation from classical mono-exponential decay of the kinetic rate processes [48]. Similar departure from mono-exponential behavior was seen in case of single-enzyme molecules in the presence of saturating concentration of substrate [10, 25]. However, our experiments with increasing substrate K^+ ion concentration suggested that physical binding of substrate K^+ ions was not sufficient to induce

dynamic disorder in hTREK1 channel (Fig. 4) unlike single-enzyme molecules [10, 33, 42].

Trichloroethanol acts as a non-essential activator of hTREK1

2,2,2-Trichloroethanol, the active metabolite of general anesthetic chloral hydrate is known to potentiate the activity of hTREK1 at whole cell and single-channel level [14, 18, 34]. Figure 5a illustrates the basal level (control) activity of the single hTREK1 channel and the activity in presence of 0.1 and 10 mM TCE. In the presence of TCE, the open probability and conductance of hTREK1

Fig. 5 Effect of trichloroethanol (TCE) on the single-hTREK1-channel activity. *i* Single channel current evoked in absence (a) and in presence of 100 μ M (b) and 10 mM TCE (c) along with their corresponding amplitude histograms (ii). The lower panel in (a, b, and c) illustrate part of the trace in the respective upper panels at high resolution. The three major conductance levels close (C), open (O), and O^* are shown by horizontal dotted bars in the current tracings. The humps in the amplitude histograms represent the major conductance levels. Note the robust increase in single-channel activity (raw data and amplitude histogram) upon TCE application

significantly increased, which have been illustrated by plotting amplitude histograms (Fig. 5b). The potentiated activity was also characterized by significantly higher number of bursts and increase in the channel occupancy in the unblocked open conductance level (Fig. 5a). Since we observed the effect of TCE on single hTREK1 in the excised inside-out configuration of patch-clamp, it is likely that TCE interacts with the intracellular face of the channel. The concentration-response of TCE activation of hTREK1 was obtained by plotting the gain in NP_o , normalized with respect to the control, against TCE concentration which was fitted to the Hill's equation. From the dose-response curve, the single-channel EC_{50} and Hill's coefficient were estimated to be $230 \pm 65 \mu$ M and 1.995 ± 0.38 , respectively, suggesting strong positive

cooperativity ($n=6-7$) [34]. Since, we often observed activity from only single channels in a patch; we attribute the observed positive cooperativity to inter-subunit interaction. From the double logarithmic plot between the pseudo-first order rate constant K_a (min^{-1}) and concentration of TCE, the reaction order associated with TCE binding was estimated to be 0.93 (~ 1.0) [34]. The reaction order of 1 with respect to the ligand suggested a single binding site for the TCE molecule to the hTREK1 channel monomer. Activators of an enzyme can be of two types: essential, without which the enzyme cannot operate, and non-essential, which enhances the basal activity of the enzyme. Since, hTREK1 shows basal level ion channel flux activity even in the absence of any modulator, TCE acts as a non-essential activator (A) of

the hTREK1 channel. This can be represented by a generalized Michaelis–Menten reaction scheme as follows:

Trichloroethanol-induced dynamic disorder in hTREK1

The activity of hTREK1 in presence of various concentrations of TCE was analyzed by estimating the flux and recording the successive waiting times. Figure 6a, c and e illustrate the single-channel close-open activity and the corresponding flux values at different concentrations of TCE. With increasing concentrations of TCE, there was progressive increase in the amplitude of flux and frequency of occurrence. The successive waiting times were plotted to obtain the waiting time histogram. Plotting the waiting times on a semi-log scale (Fig. 6b, d, and f) in absence of the TCE (control) and in presence of low concentration of TCE (10 μ M) confirmed that the waiting-time distributions were mono-exponential as shown in Fig. 7a. But, with further increase in TCE concentration, the distribution became bi- (100 μ M, Fig. 6d) and multi-exponential (10 mM, Fig. 6f). The comparison of the waiting-time distributions in log-linear scale indicates a gradual departure from mono-exponential to multi-exponential distribution (Fig. 7a). The time constants of the distributions with increasing TCE concentrations were determined by fitting the distributions with mixed exponential probability density functions (Fig. 6b, d, and f). The estimated time constants at 0, 10 μ M and 10 mM TCE concentrations have been plotted in Fig. 7b, which shows the gradual shift from mono-exponential to tri-exponential distribution. Such departure from mono-exponential distribution in presence of TCE could be explained by induction of dynamic disorder due to ligand binding analogous to single-enzyme molecules. TCE binding has been shown to induce conformational changes in proteins as evidenced by a concentration dependent blue-shift in the fluorescence emission spectra of bovine and human serum albumin [41]. Studies on the action of TCE on GABA A receptor suggests the involvement of a specific Met (M256) residue within the transmembrane domain III of the β subunit [26]. TCE has also been shown to directly interact with the hydrophobic core residues of a synthetic transmembrane α -helix bundle [22] and the transmembrane helices of the serotonin receptor [19, 40]. These evidences support our experimental observations (inside–out patch, cooperativity, and reaction order of 1.0), which suggest that the effect of TCE on the channel could be mediated by direct interaction with the channel monomers.

In a single-enzyme molecule studies, changes in the catalytic activity of enzyme molecules, attributable to conformational fluctuations, have been observed in the timescale of 10^{-4} – 10 s [10, 45]. These slow conformational changes in single-enzyme molecules manifested as distinct populations of conformers with different enzyme reactivity [10] and catalytic conversion rates. At high substrate concentration this resulted in dynamic disorder and multi-exponential distribution of waiting times. Slow conformational changes in the protein affecting its function have been well studied in allosteric regulation of enzymes, where the binding of the ligand affects a distant catalytic site [24]. A similar mechanism might operate in the case of TREK1 given the observed inter-subunit positive cooperativity. Hence, the induction of dynamic disorder in hTREK1 can be represented by the reaction scheme in Eq. 17 with n number of interconverting conformers as shown in Fig. 7c. In the schema, the ensemble of conformers bound to the substrate K^+ ion alone does not show dynamic disorder. In this condition, the conformational exchanges between the conformers are rapid enough for the catalytic rate constants (k_{1i} and k_{2i}) to be distributed randomly, thus resulting in mono-exponential distribution. In presence of low concentration of TCE (10 μ M) and high substrate concentration (150 mM), the waiting times were mono-exponentially distributed. When the substrate (K^+) concentration is saturating, the agonist (TCE) can only bind to the CS_i complex. Furthermore, at low TCE concentration (10 μ M), binding of the agonist is the rate limiting step. Hence, mono-exponential distribution in such condition can be understood by assuming a very rapid binding of TCE to the channel. However, in presence of high concentration of agonist (10 mM TCE) and high substrate (150 mM K^+) concentration, βk_{2i} becomes the rate limiting step and slow interconversion among the conformers CAS_i may result in multi-exponential decay of $f(t)$ and dynamic disorder. Quantitatively, in this condition, $f(t)$ is the weighted average of i conformers of βk_{2i} , which has been modeled by assuming three distinct populations of channel conformers (see “Materials and methods”). The reason for the choice of three interconverting conformations is the observation that at high TCE concentration (10 mM) the probability density function could only be fitted with at least three exponentials (Fig. 6f).

The quantitative expression of $f(t)$ with TCE induced dynamic disorder was obtained by numerically solving a system of five ODEs (“Materials and methods”). The simulated time courses of all the reactant and product species involved in the reaction (Fig. 7c) have been illustrated in Fig. 7d. The modeled $f(t)$ could fit the experimental data in the presence of high concentration of TCE satisfactorily, which has been illustrated in Fig. 7f (10 mM TCE) and the corresponding values of w_i in this

Fig. 6 Effect of increasing TCE (agonist) concentration on the waiting-time distribution. **a**, **c**, and **e**—*i* Single-hTREK1-channel activity recorded in the inside-out configuration in presence of 150 mM K⁺ with progressively increasing concentration of TCE (TCE **a**, 0 mM (control), **c** 100 μ M and **e** 10 mM, respectively). *ii* Ionic flux activity for the respective single-channel trace shown in (*i*). Note the change in scaling for the flux activity and the distinct “mode

switching” between long stretches of low and high flux activities at 10 mM TCE. **b**, **d**, and **f** Waiting-time distribution histograms in log-linear scale for the flux activity shown in (**a**, **c**, and **e**). The solid curve represents the probability density function (pdf) fitting the data. With increasing TCE concentration, the pdf changed from a mono-exponential (control) function to multi-exponential (10 mM) through a bi-exponential function (100 μ M)

Fig. 7 Agonist-induced dynamic disorder in single-hTREK1-channel molecule. **a** Log-linear presentation of the pdf with TCE concentrations ranging from 0 to 10 mM. Note the departure from mono-exponential distribution with increasing concentration of TCE. **b** Distribution of mean waiting times obtained from the pdf in (a). Note the increasing number of τ (time constants) with TCE concentration (data as mean $\pm$

SEM; $n=5-9$). **c** Reaction scheme depicting dynamic disorder in hTREK1 molecule in presence of the substrate K^+ and agonist TCE. **d** Simulated time course of the reactant and products involved in the catalytic cycle in (c). **e**, **f** Theoretically modeled $f(t)$ (represented by solid curve) for the experimental waiting time distribution histograms in absence of TCE (control, **e**) and in presence of 10 mM TCE (**f**)

case were 0.6804, 0.2126, and 0.1070. The involved rate constants in this simulation have been provided in Table 1. The model fit for the mono-exponential distribution as observed in the presence of only K^+ (150 mM) has been

illustrated in Fig. 7e for comparison. The distinct departure from mono-exponential distribution in the presence of the agonist is noticeable in the model fit (Fig. 7f). It suggests that the multi-exponential distribution and dynamic disorder in

Table 1 Kinetic rate constants (k) and values of other variables obtained from modeling studies

Rate constants	k_1	k_{-1}	k_{11}	k_{-11}	k_a	k_{-a}	$k_2(1)$	$k_2(2)$	$k_2(3)$	w_1	w_2	w_3	α	β
Values (s^{-1})	1×10^7	8.14×10^5	1×10^7	2.71×10^3	1.2×10^6	3.9×10^{-2}	4.57×10^4	7.3×10^2	4.4×10^3	0.62	0.183	0.097	9.0×10^{-1}	1.01×10^{-3}

presence of high concentration of TCE is indeed due to the slowly interconverting conformers with different catalytic rate constants (βk_{2i}) in the single hTREK1 channel molecule.

Memory effects in the single-channel molecule

Besides the observation of unique dynamic disorder phenomenon in $f(t)$, the turnover/flux time trace of single-channel molecule contains valuable information regarding temporal correlations between turnovers, which are not obtainable from ensemble whole cell current data. Recently, single-channel experiments on voltage-gated Na^+ channels provided evidence for such correlations in successive dwell times and previous activity-dependent molecular memory in single channels [35]. Similar memory traces have been observed in single-enzyme molecules such as β -galactosidase [10], lipase [11], and flavin oxidoreductase [33]. In order to explore such correlations in single-hTREK1-channel ionic flux activity, we evaluated the two-dimensional joint probability $g(\tau_1 \text{ and } \tau_2)$ for two waiting times τ_1 and τ_2 separated by certain number of lags (“Materials and methods”). We compared the 2D joint probability histogram for waiting times, τ_1 and τ_2 of two adjacent flux activities ($g(\tau_1 \text{ and } \tau_2)$; Fig. 8a(i)) obtained in presence of 10 mM TCE with the 2D joint histogram of the same channel for two turnovers separated by large number of turnovers (100) such that there was no correlation between them. It was described by $h(\tau_1 \text{ and } \tau_2) = f(\tau_1) f(\tau_2)$ (Fig. 8a(ii)). In the absence of dynamic disorder, these two histograms should be the same. Since the two histograms are different, it suggested the presence of a memory effect induced by dynamic disorder in the channel. To highlight the difference between the two histograms, we evaluated the difference histogram $\delta(\tau_1 \text{ and } \tau_2) = g(\tau_1 \text{ and } \tau_2) - h(\tau_1 \text{ and } \tau_2)$ as illustrated in Fig. 8a(iii). The observation of the diagonal feature in the difference histogram highlights the correlation between successive waiting times, i.e., long waiting times following long and vice versa. In order to quantify the correlation in the flux activity, we further estimated the intensity autocorrelation function $C_I(t) = \langle \Delta I(0) \Delta I(t) / \Delta I^2 \rangle$, which characterized the timescale of fluctuations in $\beta k_2(t)$ [10]. Figure 8b illustrates the autocorrellograms for the turnovers observed with progressively increasing concentrations of TCE. In the absence as well as in the presence of low concentration of agonist (10 μ M), the autocorrelation was insignificantly different from 0, suggesting the absence of any correlation (Fig. 8b). This supported our earlier observation that, in above conditions, due to lack of dynamic disorder, the rate constant $k_2(t)$ has a random and narrow distribution (mono-exponential). But with higher agonist concentration, we observed strong autocorrelation, which increasingly became multi-exponential as the dynamic disorder in $\beta k_2(t)$ became

Fig. 8 Molecular memory in the flux activity of hTREK1 channel induced by agonist. **a** 2D joint probability distributions of waiting times (τ_1 and τ_2) adjacent to each other, $g(\tau_1, \tau_2)$ (i) and at a larger separation, $f(\tau_1) f(\tau_2)$ (ii) obtained from the time trace of a single hTREK1 channel in presence of 150 mM K^+ and 10 mM TCE. The color code represents the occurrence (z -axis) from 50 (deep red) to 1 (dark blue). **iii** The difference 2D histogram, $\delta(\tau_1 \text{ and } \tau_2) = g(\tau_1 \text{ and } \tau_2) - f(\tau_1) f(\tau_2)$. The pixels in “cold” colors (less probable) gather along two wings in the x - y plot, whereas those with “warm” color (more probable) are mainly spread around the diagonal. The time-axes

(τ_1 and τ_2) in (i, ii, and iii) run from 0 to 35 ms. **b, c** Autocorrelation functions obtained from hTREK1 flux activity traces for the intensity (**b**) and waiting times (**c**) obtained in the presence of increasing concentrations of TCE (0, 0.1, 1, and 10 mM). The autocorrelation in absence of TCE (control) is flat (gray) and with increasing concentration of TCE the autocorrelation increases steadily and persists for progressively long time. The dotted line depicts the best fit as per Eq. 18 in the text. The β values for the fits are 0.43, 0.26, and 0.17 for 0.1, 1, and 10 mM TCE, respectively

more apparent (Fig. 8b). The strong autocorrelation in the data was exemplified by the distinct clustering in the turnover/flux events in presence of high concentration of the agonist (10 mM TCE) (Fig. 6e), where periods of high flux activity was separated by long stretches of low activity, i.e., higher activity often followed higher, than lower flux activity and vice versa. Correlation in the flux activity thus results in distinct mode switching in single-channel activity. On the other hand, in the absence of the agonist, the high and low activities were randomly interspersed (Fig. 4a, c, and e). Similar persistent mode switching has been reported in case of the HCN channel [4]. It has been proposed to impart a short-term activity-dependent memory to HCN channel, which could modify synaptic integration and contribute to regulation of rhythmic firing [9, 31, 43]. Therefore, TCE induced mode switching behavior might have important physiological significance.

In order to further verify the memory effect, we also obtained the autocorrelation function of the waiting times

(τ), as described earlier [10]. Figure 8c illustrates the autocorrellogram for the waiting times, which shows that in absence of the agonist and in presence of low concentration of TCE, $C_\tau(m) = 0$ when $m > 0$. With increasing concentration of TCE, however, there was progressive development of autocorrelation similar to that in case of intensity of flux. It has been shown earlier that $C_\tau(t)$ is approximately the normalized autocorrelation function of $k_2(t)$, i.e., $\langle \delta k(0) \delta k(t) \rangle / \langle \delta k^2 \rangle$ under the limit of slow fluctuations of $k_2(t)$ [10, 46]. Therefore, we suggest that the observed correlation in the ion channel flux is indeed the result of strong correlation in the catalytic rates, $k_2(t)$. The relaxation of the autocorrelation functions were highly multi-exponential and was fitted to stretched exponential functions similar to that used earlier [10], given by :

$$C_I(t) = C_I(0) \exp \left[- \left(\frac{t}{t_0} \right)^\beta \right] \quad (18)$$

with $\beta = 0.21 \pm 0.06$ ($n=5$) for 10 mM, whereas for 100 μ M TCE, the β was 0.53 ± 0.1 ($n=8$). We observed low values of β ($\beta < 1$) for agonist concentrations higher than 100 μ M for a total of 11 different patches. In all cases, at highest concentrations of TCE (10 mM), the autocorrelation persisted up to 10^4 lags (Fig. 8b, c). This highlights the broad range of timescales of $k_2(t)$ fluctuations, spanning at least 10^{-2} –100 s in real time. A broad range of conformational fluctuations in protein molecules occur in these time scales [33]. It strongly suggests that the fluctuations that we observed in the catalytic rates of ionic efflux in case of hTREK1 do indeed originate due to the conformational fluctuations upon agonist binding.

The concentration of TCE used in the experiments is in the range found in clinical conditions after administration of chloral hydrate [37] and has been discussed earlier [14]. TCE has been strongly implicated in the shorter form of anesthetic preconditioning mediated neuroprotection, which sets in within few seconds of TCE exposure and persists up to 2 h [5, 47]. Since hTREK1 is one of the major direct targets of TCE and exhibits the memory-driven mode switching in activity; it might be an important candidate for anesthetic preconditioning. But more detailed work needs to be done to prove this plausible hypothesis. The concentrations of TCE used here that induces the memory phenomenon in TREK1 channels, could be clinically potent concentrations. In essence, the hTREK1 channel exhibits non-stochastic, deterministic behavior in its rate processes, at least in the presence of a high concentration of agonist. However, the observed memory effect in the single hTREK1 molecule might not be significant in a system comprising of large number of molecules due to ensemble averaging, but when a small number of channel molecules are available for activation, the effect of such memory effect will be readily realized [10].

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