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Use of conditional distributions in the analysis of ion channel recordings

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Abstract. A method to test the Markov nature of ion channel gating is proposed. It makes use of singly and doubly conditional distributions. The application of this method to recordings from single BK channels provides evidence that at least two states of the underlying kinetic scheme are left at a constant rate. Moreover, the probabilities, when leaving a state, of reaching another given state are shown to be constant for all the states of the system.

Key words: Ion channels – Markov processes – Conditional distributions

Introduction

Ion channels have usually been modelled as Markov systems with a few discrete states. In Markov systems the transition rates for leaving each state are assumed to be independent of the time spent in that state and of the way that state was reached. There are two classes of Markov systems: those which do not allow transitions between states with the same conductance and those where such transitions, which we will call "hidden" as they cannot be detected directly on the recording, can occur. These systems have been described as uncoupled and coupled respectively (Kienker 1989).

It is possible to show analytically that, for uncoupled Markov systems and for Markov systems where the detailed balance holds (i.e. in thermodynamic equilibrium), the experimental distributions of open and closed intervals are linear combinations of decreasing exponentials, of the same number as the number of open and shut states respectively (Colquhoun and Hawkes 1981, 1983).

The distributions of either open or closed dwell times from identified ion channels have been reported in several papers and kinetic models accounting for them have been proposed. However, these distributions (also referred in the following as global distributions) do not generally hold all the information contained in the time sequence of openings and closings, because the possible correlation between intervals is lost. The analysis of auto- and cross-correlation functions has been used to partially recover the correlation information (Fredkin et al. 1985; Labarca et al. 1985; Colquhoun and Hawkes 1987) but only the bidimensional distributions of the pairs of adjacent open and closed intervals have been shown to preserve all the information for Markov systems (Fredkin et al. 1985; Steinberg 1987; Bauer et al. 1987). Only recently has the analysis of experimental data been tackled using bidimensional and conditional distributions (McManus and Magleby 1989). Conditional distributions are obtained by extracting subsets which satisfy given conditions from the whole set of open or closed dwell times: typical conditions are that the values of preceding dwell times belong to given ranges. As for the content of the information conditional distributions are equivalent to bidimensional distributions, but the former are easier to visualize.

The theoretical description of ion channels as Markov systems has recently been debated, suggesting, in particular, that the probability of leaving a state decreases as the time spent in that state increases (Liebovitch and Sullivan 1987; Läuger 1988; Milhauser et al. 1988; Liebovitch 1989). If this holds, the fit of experimental distributions by sums of exponentials has no physical interpretation.

This paper aims to show that the conditional distributions, when applied to experimental data, can test the physical meaning of the fitting components, as well as the fact that the system's evolution is independent of its past activity. Preliminary results have already been published in abstract form (Barbi et al. 1988, 1989).

Dwell time distributions, conditioned by limiting the duration of the preceding intervals, have already been used in a recent paper (McManus and Magleby 1989) as a test for the Markov hypotheses. Here we employ quite a different approach, which involves double and/or second order conditions for the distributions, and propose it as a more suitable test of Markov hypotheses.

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Description of the method

Theoretical background

Our analysis aims to test if a set of experimental data matches the Markov hypotheses.

First of all let us define the tools used in the analysis. The *first-order conditional distributions* are those obtained by selecting the dwell times preceded by intervals in given ranges, while the *second-order conditional distributions*, also referred to as closed-closed or open-open distributions, are those obtained when subsets of openings or closings are selected, by imposing a condition on the preceding interval of the same kind.

The simultaneous application of first and second-order conditions gives the *doubly conditioned distributions*.

The general theory of discrete Markov systems, developed by Colquhoun and Hawkes (1981, 1983, 1987) and by Fredkin et al. (1985), yields for the conditional distribution $\Omega(t, T)$ of closings preceded by openings longer than T:

$$\Omega(t, T) = \sum_{1}^{N_c}{}_j P_j(T) \exp(-t/\tau_j), \tag{1}$$

with

$$P_j(T) = \sum_{1}^{N_0}{}_i Z_{ij} \exp(-T/\theta_i), \quad j = 1, \ldots, N_c. \tag{2}$$

N_c and N_0 being the numbers of closed and open states respectively, and τ_j and θ_i their time constants.

These expressions, which are easy to obtain for uncoupled schemes (see Appendix) hold for coupled schemes too.

In the following we will always refer to the conditional distributions of closings but similar equations and conclusions apply to the openings.

Expressions (1) and (2) show that, for Markov systems, the conditional distributions contain the same exponential components which fit the corresponding global distributions and that the relative contribution of each closed state depends on the imposed condition. More precisely, $P_j(T)$, the fraction of closings which belong to the component characterized by τ_j in the conditional distribution of closings, is a linear combination of the exponentials fitted to the distribution of openings. It is important to realize that there is a one-to-one correspondence between the shape of the conditional distributions and the set of weights $P_j(T)$ of the contributing states.

Testing Markov hypotheses

Expressions (1) and (2) above can be used to test if the investigated system satisfies the Markov hypotheses and non-Markov systems which do not fit such expressions can easily be imagined (Barbi and Petracchi 1990; McManus and Magleby 1989). Nevertheless, verifying them partially or even totally is not enough to prove that the Markov hypotheses are satisfied. For instance, the fact that the conditional distributions are fitted with the same exponential components which fit the global ones, though compatible with the Markov nature of the system (McManus and Magleby 1989), is far from being a proof of it. An example of a non-Markov system where the same exponentials fit the global as well as the conditional distributions was given by Barbi and Petracchi (1990).

What characterizes a Markov process is that the transition rates between states are constant or that, in a perfectly equivalent way: i) the probability per unit time of leaving a state is independent of both the time spent in it and the way it was reached, ii) the probability P_{ij} when leaving state **i** of reaching state **j** is a constant. In terms of the rate constants k_{ij}, the P_{ij} are given by:

$$P_{ij} = k_{ij}/\sum_j k_{ij}$$

and can be constant also when the k_{ij} vary with time. In the Appendix we analyse a system where the probability of leaving a state depends on the time spent in it, but the P_{ij} are constant.

Let us consider separately how to test point i) and point ii) of the Markov hypotheses on the observed system.

Point i) implies that the components of the dwell times distributions are exponentials.

In the following we will use the expression "physical components", for instance of the closings, for the distributions of the dwell times which start in a given closed state. Thus, the exponentials in a discrete and uncoupled Markov system and the functions (3a) for scheme 1 of the Appendix are physical components. We use the expression "physical components" because these functions depend on the structure of the system.

The problem of testing point i) is the same as identifying the physical components and checking they are exponential, and it can be dealt with empirically by using first and higher order conditional distributions. What is required for a single physical component is that it evolves as a unit, i.e. the dwell times in each subset of it are followed by dwell times exhibiting the same distributions.

Let us describe the procedure we follow to identify, when possible, the physical components. Let us suppose that applying two different conditions A and B to previous openings yields two conditional histograms of closings, with different shapes, that we call A_1 and B_1. This means that the global histogram of closings is a composite one and can be split into components. Now consider one of these two histograms, for instance histogram A_1, and examine what is the fate of the closings falling on its right or on its left side. Two doubly conditioned histograms of openings are obtained, A_2 and B_2, which correspond respectively to condition A and "previous closing longer than T" and to condition A and "previous closing shorter than T". If A_2 and B_2 still have different shapes the procedure can be repeated. When, on the other hand, a histogram is obtained which cannot be split into subsets with a different fate, it is a candidate for a physical component.

A priori there is no guarantee that physical components can actually be isolated; it depends on the underlying kinetic scheme. For instance, if there is no correlation in the sequence of openings and closings there are no methods to identify physical components. In this and in other cases it is impossible to distinguish between a complex Markov scheme and a simpler one with physical components broader than an exponential (see Appendix). Anyway, if in the analysis of experimental data the use of suitable conditions allows a single exponential component to be isolated from a complex histogram, then the existence of a state characterized by a constant probability of leaving it can be assumed.

A more quantitative approach might be tried by considering the dependence on the conditioning parameter of the weights of the exponential components fitted to the conditional distributions and by fitting it to expression (2) (Petracchi and Barbi 1990). However, this approach is very difficult when applied to experimental data.

To test point ii) of the Markov hypotheses a general approach is suggested in Fredkin et al.'s paper (1985). Again we take advantage of the use of doubly conditioned or second-order conditional distributions. For Markov systems, these distributions are determined by the first-order ones (Fredkin et al. 1985). This statement does not depend on the form of the components but only on the constancy of the probability P_{ij} of reaching state **j** when leaving state **i**. Therefore, it holds for Scheme 2 of the Appendix.

How can Fredkin's theorem be used in a practical way? What characterizes a process with constant P_{ij} is that the next state to be reached does not depend on the dwell time in the current or in previous states, but only on the current occupied state. Moreover, the condition on the previous dwell time does not determine the state occupied by the system, but constrains the system in a combination of states, which is characterized by their weights (the $P_j(T)$ which appear in expressions (1) and (2)). Thus, unless the system is degenerate[1], there is a one-to-one correspondence between the occupancy probabilities of each state, which are functions of the imposed condition, and the shape of the first-order conditional histogram. Therefore, if the combination of states determined by a condition A yields the same first-order conditional histogram as a different condition B does, the shapes of the second-order conditional histograms will also coincide, owing to the coincidence of the combinations of states corresponding to the intermediate histograms. In particular, if a first-order conditional histogram scales with the corresponding global one, so will the second-order conditional histogram. Such a condition is often found and this is a practical way to test Fredkin's theorem on the analysed system, and point ii) of Markov hypotheses.

Application to experimental data

Materials and methods

The cell free patch clamp technique (Hamill et al. 1981) was used to study one type of potassium selective ion channel. Single calcium-activated BK channels from mouse EC-1003 cells (Simonneau et al. 1985) were recorded. All the experiments were carried out at room temperature (19–21 °C), by using the inside-out configuration in symmetrical potassium solution (millimolar composition: KCl 120, $MgCl_2$ 1, $CaCl_2$ 0.1, HEPES KOH 10, pH 7.3). Single channel data were stored on a modified videotape recorder (Bezanilla 1985) and kinetic analysis was performed after low-pass filtering at 3 kHz. State transitions were converted, by a two-level window discriminator, into a digital signal, which was directly fed into an IBM-XT computer via a commercial I/O card. Visual control was performed throughout the acquisition. Open and closed lifetime distributions were plotted as log-binned histograms (Sigworth and Sine 1987) and multi-exponential functions were fitted to them. The quality of the fits was checked by computing the χ^2.

Results

The method of conditional distributions was applied to experimental data collected from single BK channels recorded in the mouse EC-1003 cell line.

Figure 1 illustrates global dwell time distributions for openings and closings. The distribution of open times has been fitted by the sum of three components with time constants 0.07, 0.4 and 2.4 ms (Fig. 1 a), while four exponential components at 0.075, 0.46, 11 and 210 ms have been used to fit the global distribution of closed dwell times (Fig. 1 b).

Figure 2 shows first order conditional distributions obtained by restricting the preceding intervals to given subsets. In particular, Fig. 2a gives the distributions of openings preceded by closings which are longer than 100 ms or shorter than 0.2 ms and Fig. 2b those of closings preceded by openings longer than 2 ms or shorter than 0.15 ms. As already found by McManus et al. (1985) for different channels, an inverse relationship holds between the durations of adjacent open and closed intervals. On the other hand, the distribution of openings preceded by closings with an intermediate range duration (0.3–20 ms) has the same shape as the global distribution (Fig. 2c) and the same occurs for the closed time distribution (Fig. 2d).

For comparison Fig. 3 shows the conditional distributions obtained by McManus and Magleby (1989) for the BK channel of rat myotubes, plotted using the same representation as for our data. The values of time constants and weights come from Figs. 2 and 5 of the quoted paper. In our histograms the conditions used yield a significant modification on the distributions of the subsequent dwell times while a weaker effect is observed in the histograms reported by McManus and Magleby. This may be ascribed to the different channels studied and/or to the choice of the conditions.

The histograms plotted in Fig. 4 show second-order conditional distributions: here subsets of openings or closings are selected by imposing a condition on the preceding interval of the same kind. Figures 4a and c plot the histograms of closed intervals selected on the basis of the duration of the preceding *closed* interval, while an open-

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[1] Degenerate system: one where two or more components, of openings or closings, coincide

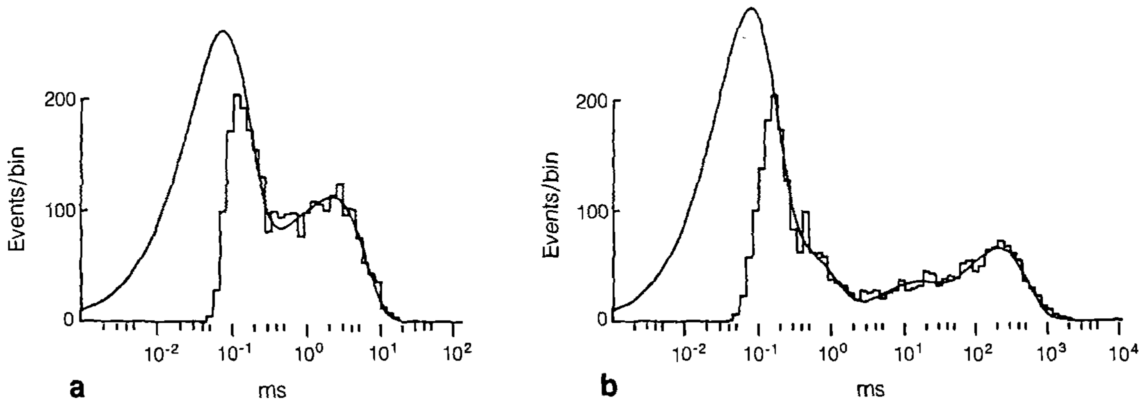


Fig. 1a, b. Global distributions of open (**a**) and closed (**b**) dwell times measured on experimental data recorded from a BK channel of mouse EC-1003 cells; 6 000 events were processed. The time constants and percent weights of fitting curves are: 0.07 ms (63%), 0.4 ms (8%) and 2.4 ms (29%), for openings; 0.075 ms (64%), 0.46 ms (14%), 11 ms (6%) and 210 ms (16%), for closings

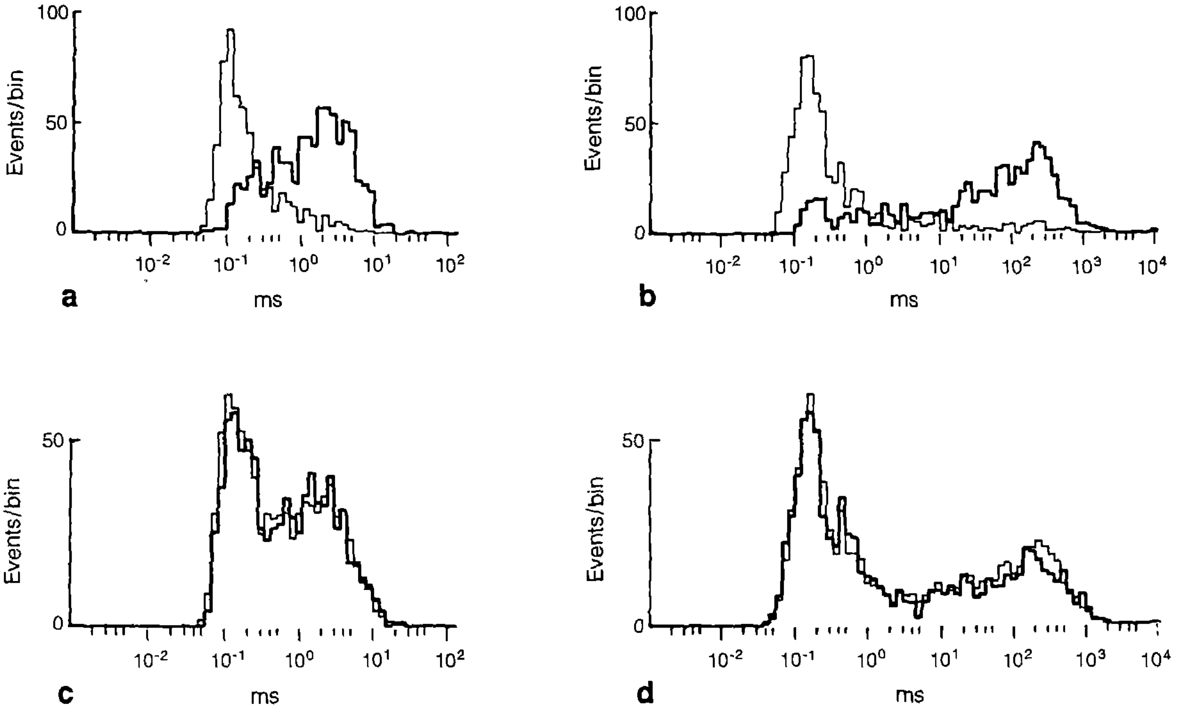


Fig. 2a–d. Conditional distributions of: **a** openings preceded by closings longer than 100 ms (*thin line*) or shorter than 0.2 ms (*thick line*); **b** closings preceded by openings longer than 2 ms (*thin line*) or shorter than 0.15 ms (*thick line*); **c** openings preceded by closings in the range 0.3–20 ms (*thick line*); **d** closings preceded by openings in the range 0.2–1 ms (*thick line*). Distributions in **c** and **d** were scaled by 3.3 on the ordinate and superimposed on the corresponding global ones (*thin line*). Same data as Fig. 1

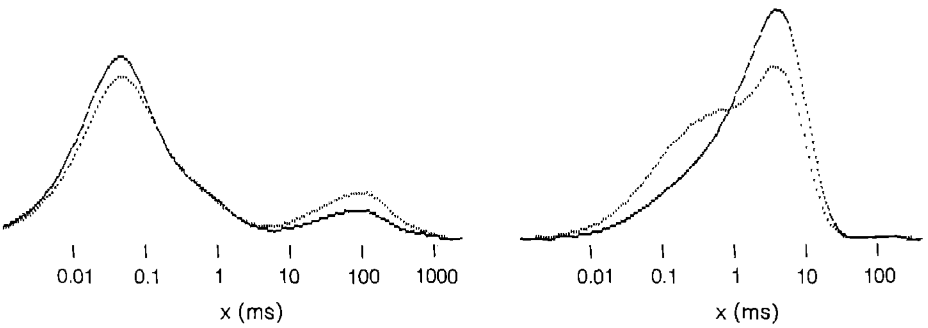


Fig. 3. Conditional distributions obtained by McManus and Magleby (1989) in the BK channel of the rat. *Left*: Closings preceded by openings which are longer (*thin line*) or shorter than 0.7 ms (*thick line*); *right*: openings preceded by closings which are longer than 3 ms (*thin line*) or shorter than 0.15 ms (*thick line*)

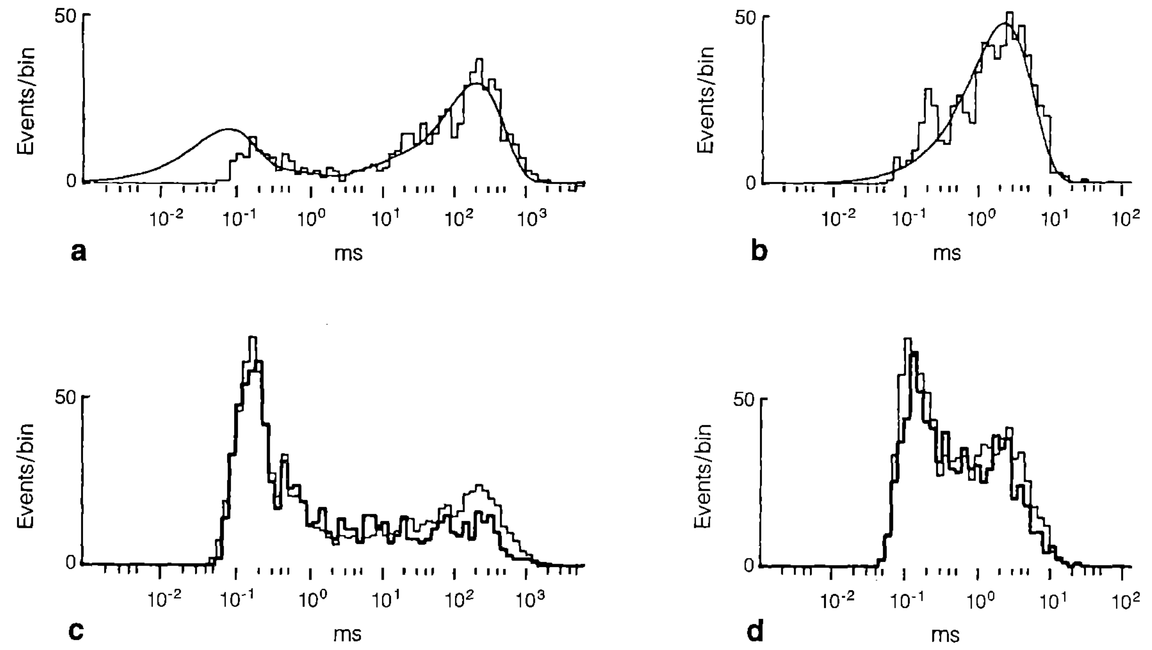


Fig. 4a–d. Histograms of: **a** closings preceded by closings longer than 100 ms; **b** openings preceded by openings longer than 2 ms; **c** closings preceded by closings in the range 0.3 ms to 20 ms; **d** openings preceded by openings in the range 0.2–1 ms. Distributions in **c** and **d** were scaled by 3.3 on the ordinate and superimposed on the corresponding global ones (*thin line*). Weights of exponential components of the fitting curve in **a** are: 32% (for the component at 0.075 ms), 7% (at 0.46 ms), 6% (at 11 ms) and 55% (at 210 ms). Same data as Fig. 1

open condition holds for Fig. 4b and d. The dwell time limitations used to obtain second-order histograms of openings (closings) in Fig. 4 are the same as those used for first-order histograms of closings (openings) in Fig. 2. Thus the openings selected in Fig. 4b and d (with an open-open condition) arise after the closings reported in Fig. 2b and d respectively, and similarly, the closings selected in Fig. 4a and c (with a closed-closed condition) arise after the openings of the corresponding histograms in Fig. 2.

Figure 4 shows that, depending on the condition imposed, quite different second-order distributions are obtained. For the cases of closings and openings in Fig. 4c and d, respectively, the second-order distributions scale with the corresponding global ones and the same holds for the first-order distributions obtained with the same condition (Fig. 2c and d). While the second-order distributions reported in Fig. 4a and b differ from the global ones as well as happens for the first-order distributions obtained with the same condition (Fig. 2a and b).

In the histograms of Fig. 4a and b the slowest exponential component (2.4 ms for the openings and 210 ms for the closings) is greatly enhanced compared to the corresponding one in the global distribution. In particular, the histogram in Fig. 4b can be fitted by the single exponential with a time constant of 2.4 ms.

We note that the 210 ms component in the distribution of closings is quite far away from the nearest component, (11 ms time constant) and the same occurs for the longest component (2.4 ms) of the distribution of openings. So we can compare, for instance, the distributions of the closings preceded by openings from different subsets of the 2.4 ms component, as done in Fig. 5b. Here histograms plotted with thin and thick lines, scale almost perfectly and the thin line histogram corresponds to the subset of openings in the 1–2.5 ms range, while the thick line histogram derives from the subset with openings which are longer than 2.5 ms. Analogously, as shown in Fig. 5d, the restriction of closings to different subsets of the 210 ms component selects successive openings with the same distribution. This analysis provides evidence for a physical meaning of the longest components of the distributions of openings and closings.

Further evidence of the real existence of the 210 ms component of closings is given in Fig. 6. Here different subsets of this component are obtained by applying conditions on the previous opening (Fig. 6a), and the histograms of the openings which follow the long lasting (>70 ms) closings from these subsets are plotted in Fig. 7b. They still have the same shape. On the other hand, the doubly conditioned distributions of openings which follow the closings from the two subsets of Fig. 6a and falling in the range 0.3 ms to 20 ms have clearly different shapes (Fig. 6c).

Lastly, it is worth noting that identical conditional histograms were obtained by using the same conditions on the following intervals as on the preceding ones, which implies that the data satisfy microscopic reversibility.

Discussion

Here we have presented the most rigorous test yet performed for Markov gating. In principle one could try to do something more. In fact, as shown elsewhere (Petracchi and Barbi 1990), the use of expressions (1) and (2) on data obtained from uncoupled Markov systems should allow the kinetic scheme to be identified, while the

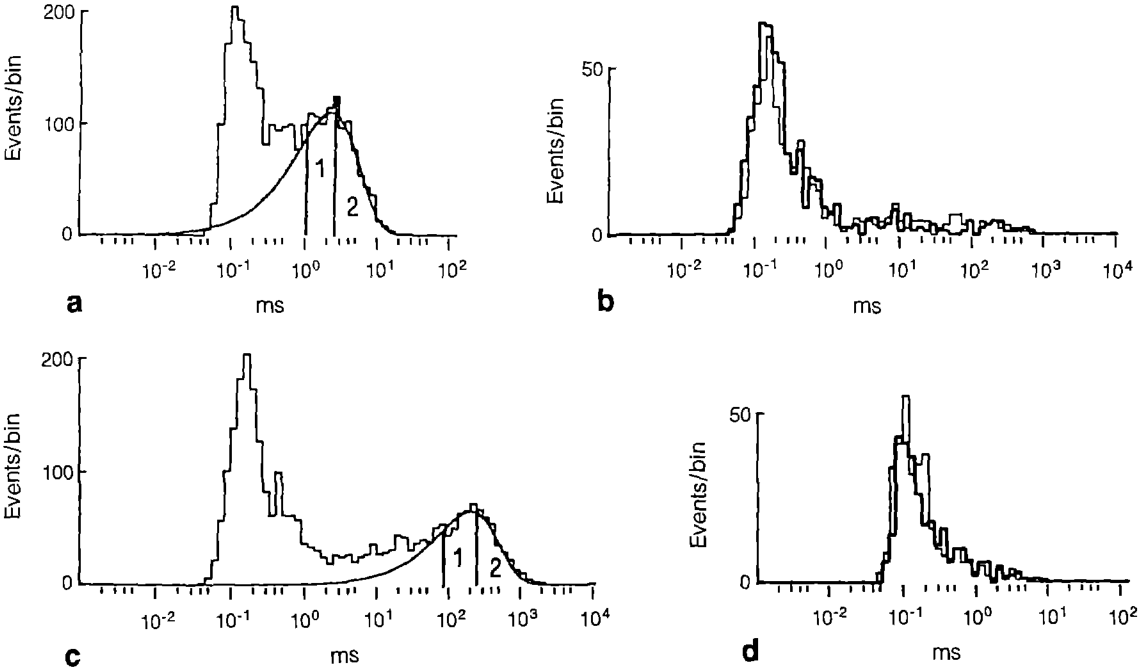


Fig. 5. **a** and **c** Global distributions of openings and closings with the exponential components at 2.4 ms and 210 ms respectively shown; **b** and **d** conditional distributions of closings (*openings*) preceded by openings (*closings*) belonging to subset 1 (*thick line*) or 2 (*thin line*) of histograms on the left. Same data as Fig. 1

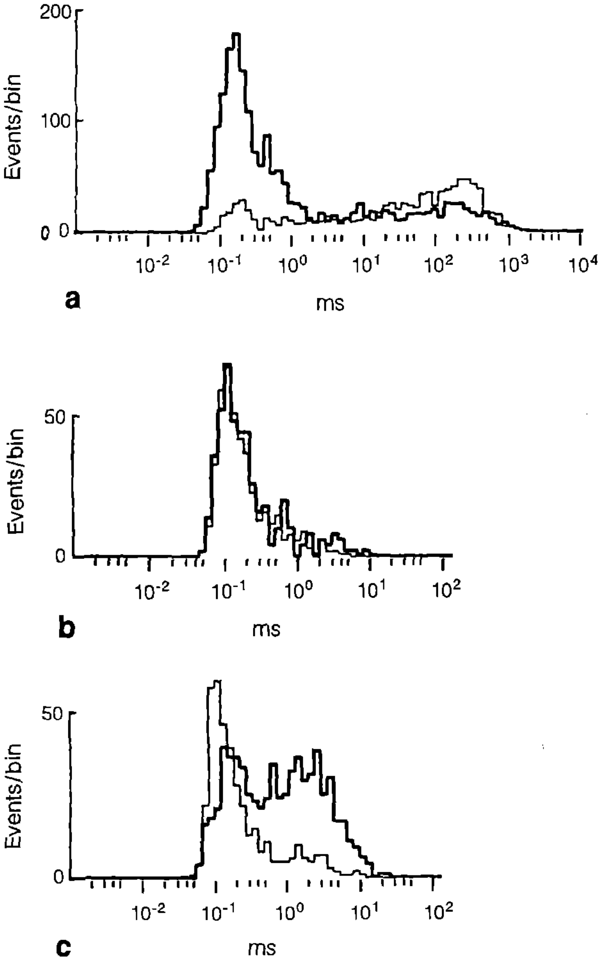


Fig. 6. **a** Distributions of closings following openings longer (*thick line*) or shorter than 0.2 ms; **b** and **c** doubly conditioned distributions of *openings*, see text for details. Same data as Fig. 1

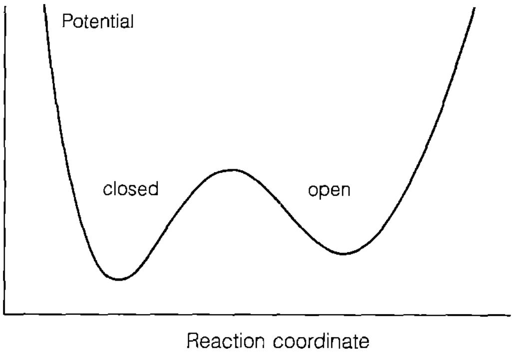
coupling within the aggregates would be signalled by negative coefficients in expression (2). Thus, one way to control if one channel's activity matches the Markov hypotheses, might be to get the kinetic model by fitting expressions (1) and (2) and the equivalent ones for the openings to the experimental data, and *then to compare* the distributions obtained with *multiple conditions* from the model and from the experimental data.

Unfortunately, this approach is very limited. In fact, the distributions obtained by analysing patch clamp recordings are deeply affected by the loss of brief events due to signal filtering. The problem has been investigated in detail (see for instance Roux and Sauvè 1985; Blatz and Magleby 1986; Crouzy and Sigworth 1990; Hawkes et al. 1990), but a general method to obtain the kinetic scheme from noisy and band limited data has not yet been used.

The reconstruction of the kinetic model might also be tried, as recently suggested by Magleby and Weiss (1990a), by using an iterative method of simulating the model with its noise and filtering, and comparing simulated and experimental data. Until now this method, which would certainly be the best, has only been applied to simulated data (Magleby and Weiss 1990b). The method used in the present work, in order to test the Markov hypotheses, is simpler and only involves analysing, together with first-order conditional distributions, doubly conditioned or second-order conditional distributions.

The results reported herein for this BK channel satisfactorily match what is expected from a Markov process. Suitable conditions on previous intervals select dwell times whose distribution is well fitted by a single exponential component, which is the longest one both for openings and for closings (Fig. 4a and b); and the histograms of intervals following the dwell times from different subsets of this component can be superimposed after scaling (Figs. 5 and 6 a). In other words, each subset of the longest component generates the same histogram of the following intervals, which implies that this component evolves as a unit. Moreover, open-open and closed-closed conditions are only effective in producing different histograms when they affect the distribution of interposed time intervals (Figs. 2 and 4). This also applies when multiple conditions are used (see Fig. 6c and the different shapes of the two histograms in Fig. 6 a). In other words, the system's evolution only depends on the combination of states currently occupied by the system.

From a physical point of view it is obvious that, if the system's open and closed states correspond to defined energy levels, the probability of leaving them is constant. On the other hand, if a functional state corresponds to a set of energy sublevels, the assumption of constant transition probabilities cannot be taken as generally true. The idea that the protein state moves in an almost continuous domain has often been suggested, particularly for the interpretation of spectroscopic data (Frauenfelder et al. 1988). In the limit case, for a classical system moving in potential wells such as those shown in Scheme 1 below, it is clear that the transitions from left to right and vice versa depend on the thermal interactions with the medium and on the motion equations. It is not easy to predict the analytical shape of the dwell time distribution. By computer simulations we have seen that it is generally not exponential and that the only way to obtain an exponential-like shape is to increase the energy barrier until it is very high compared to the mean value of thermal energy.



Scheme 1

In this context, the results obtained here can be described by saying that in this BK channel the longest time constants correspond to functional states with given energy levels. For the other components we cannot affirm that they correspond to simple energy levels or, equivalently, are left with constant probability. In fact, we have not separated single exponentials with short or intermediate time constants. Nevertheless Figs. 4–6 show that the evolution of the system only depends on its actual state (or combination of states). For these components we have proved that the probability P_{ij} of reaching state **j** when leaving state **i** is constant, whereas for the components with the longest time constants we have also proved the constancy of the rate constants for leaving these states.

Appendix

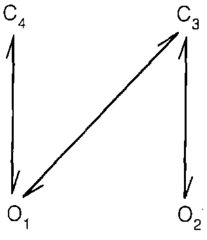
Here we compute the expressions of the conditional distributions both for uncoupled Markov models and a particular non-Markov one.

For uncoupled Markov models the conditional distribution $\Omega(t, T)$ of closings preceded by openings longer than T is obtained by simply applying compound probabilities and is given by:

$$\Omega(t, T) = \sum_{1}^{N_c}{}_{j} \sum_{1}^{N_0}{}_{i} [A_i \exp(-T/\theta_i)] P_{ij} 1/\tau_j \exp(-t/\tau_j), \tag{1 a}$$

where N_0 and N_c are the numbers of open and closed states respectively, θ_i and τ_j are their time constants, A_i is the weight of the open state **i** and P_{ij} is the probability of reaching state **j** when leaving state **i**. The product inside square brackets represents the tail distribution of the dwell times in the open state **i**, i.e. the fraction of openings belonging to state **i** and longer than T. The product of this number by the transition probability to state **j** (P_{ij}) and by the probability density of a sojourn of duration t in state **j** ($1/\tau_j \exp(-t/\tau_j)$), summed over all states, just gives the probability density of a closing lasting t and following an opening longer than T.

Now let us examine a model, where the probability K_i of leaving state **i** per unit time decreases as time spent in state **i** increases. In Scheme 2 below we assume that this occurs for all the states.



Scheme 2

According to Liebovitch and Sullivan (1987) we write:

$$K_i = B_i t^{1-D_i} \qquad 1 < D_i < 2. \tag{2a}$$

Parameters B_i and D_i are characteristic of each state, like the time constants in a Markov system. The time dependency of K_i determines the distribution of the dwell times in state **i** given by (Liebovitch and Sullivan 1987):

$$f_i(t) = B_i t^{1-D_i} \exp(-B_i t^{2-D_i}), \qquad 1 < D_i < 2. \tag{3a}$$

These distributions may span over several decades and may be very broad when compared to a single exponential (an example of such a function is given in Fig. 7). The system is thoroughly defined by assigning to each state, besides B_i and D_i, the probabilities P_{ij}, which are assumed constant, of reaching state **j** when leaving state **i**.

The conditional distribution of closings following openings longer than T is given by:

$$\Omega(t, T) = A_1 F_1(T)(P_{13} f_3(t) + P_{14} f_4(t)) + A_2 F_2(T) P_{23} f_3(t), \tag{4a}$$

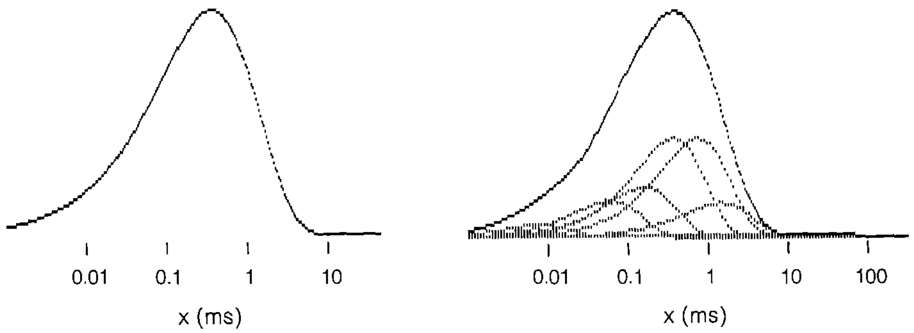


Fig. 7. *Left*: Plot of the function $f(t)=B\,t^{1-D}\exp(-B\,t^{2-D})$, for $D=1.3$ and $B^{1/(D-2)}=0.63$ ms. *Right*: Plot of the linear combination of exponentials fitted to it. Each exponential is plotted by a *thin line*. Time constants and weights of the exponentials are respectively: 1.4 ms (0.1), 0.75 ms (0.3), 0.37 ms (0.3), 0.16 ms (0.15), 0.06 ms (0.11), 0.01 ms (0.04)

where A_1 and A_2 are the fractions of openings occurring in states 1 and 2 respectively, and $F_1(T)$ and $F_2(T)$ are the probabilities of openings longer than T in O_1 and O_2 $(F_i(T)=\int_T^\infty f_i(t)\,\mathrm{d}t)$.

The method of computing $\Omega(t, T)$ for this model is the same as for an uncoupled Markov system; expression (4a) reduces to (1a) after replacing f_3 and f_4 by the normalized exponential components $1/\tau_j \exp(-t/\tau_j)$ and the tail distributions of the open states $F_1(T)$ and $F_2(T)$ by the tail distributions of exponential components.

Now, the global distribution of closings coincides with $\Omega(t, T)$ for $T=0$ and so it contains the same non-exponential components $f_3(t)$ and $f_4(t)$ which appear in the conditional distributions. Thus, since each $f_j(t)$ can be fitted by a combination of exponentials (see Fig. 7), the same exponentials fit both global and conditional distributions. We must stress that f_3 and f_4 are the physical components of the histogram, while the decomposition of the histogram in exponential functions is a mere computing trick with no physical meaning. Nevertheless, also for such non-Markov systems with physical components broader than a single exponential, conditional distributions can be fitted by sums of the same exponentials which fit the global ones.

Even considering the weights of the exponential components in conditional distributions, we cannot discriminate straightforwardly between Markov systems and systems of the class discussed above. In fact, if the system is Markovian, the weights of each components of the conditional distribution of closings vary with T as a linear combination of the exponential components of openings (see Eq. (3)) but this occurs for the system of Scheme 2 as well (see Eq. (4a) and substitute for $F_1(T)$ and $F_2(T)$ the corresponding combination of exponentials). What is important to realize is that in this case the weights of all the exponentials fitted to the physical components $f_3(t)$ or $f_4(t)$ depend in the same way on T.

In conclusion, a Markov system which is indistinguishable from Scheme 2 can be conceived (one need only to substitute each state in Scheme 2 with a suitable combination of Markov states); but, for Scheme 2 and equivalent ones, it would be impossible to separate or significantly enhance a single exponential component from a complex histogram.

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