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Hurst Analysis Applied to the Study of Single Calcium-activated Potassium Channel Kinetics

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The gating of ion channels has been modeled by assuming that the transitions between open and closed states is a memoryless process. Nevertheless, analysis of records of unitary current events suggests that the kinetic process presents short-term memory, i.e. the open- and closed-dwell times are short-term correlated. Here the rescaled range analysis (R/S Hurst analysis) is used as a method to test long-term correlation, in single calcium-activated potassium channels present in Leydig cells. The Hurst coefficients, calculated for four different voltages (V) are: 0.634 ± 0.022 ($n = 3$) for $V = +20$ mV; 0.635 ± 0.012 ($n = 4$) for $V = +40$ mV; 0.606 ± 0.020 ($n = 4$) for $V = +60$ mV and 0.608 ± 0.026 ($n = 4$) for $V = +80$ mV. This indicates that open- and closed-dwell times are long-term correlated and do not change with the voltage applied to the patch at a 5% significance level ($F = 2.2402$; $p = 0.140715$). Randomly shuffling the experimental data removes the correlation in all voltages. When the Hurst method was applied to the results from a simulated three-state Markovian model, it showed that it could not account for the long-term correlation found in the experimental data. In this case, H has the following values: 0.5498 ± 0.018 ($n = 100$) for $V = +20$ mV; 0.5557 ± 0.0202 ($n = 100$) for $V = +40$ mV; 0.5565 ± 0.0246 ($n = 100$) for $V = 60$ mV and 0.5595 ± 0.0247 ($n = 100$) for $V = +80$ mV. Even a four-state Markovian model was not adequate to correctly simulate the long-term memory found experimentally, with H values significantly different from those found for the experimental data, in the same voltage range ($F = 15.0355$; $p = 0.00001$). In conclusion, this paper shows that: (1) the open- and closed-dwell times in the single calcium-activated potassium channel of Leydig cells are long-term correlated; (2) Three- and four-state Markovian models, which describe very well the dwell time distributions, are not adequate to describe the long-term correlation found between the open and closed states of this ion channel.

Introduction

The dissipative movement of ions across the cell membrane is made through hydrophilic pathways physically identified with proteins that span

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the lipid bilayer. These proteins form channels which are characterized by their selectivity and by making transitions between an open and a closed conformation during their functioning. The switching of ion channels between different states is brought about by energy derived from thermal fluctuations, the electrical field and/or binding of ligands. The sequence of open and closed states can be measured in individual channels by using the patch-clamp technique to record the ionic current flowing through a single channel (Hamill *et al*., 1981). Models used in the study of ion channel kinetics usually assume that the protein can dwell only in a few discrete states. The switching between these states is considered random and the probabilities per unit time (rate constants) of transitions between them depend only on the present state, and not on the previous history of the channel (Colquhoun & Hawkes, 1981, 1982). However, short-term correlation in experimental and simulated data has been shown to exist in some channels (McManus *et al*., 1985; Ball & Sansom, 1988; Colqhoun & Hawkes, 1987; Steinberg, 1987; Labarca *et al*., 1985; Jackson *et al*., 1983) and this can be interpreted to indicate that the channel kinetics is a process with short-term memory. The studies of Toib *et al*. (1998) have shown direct experimental evidence for the existence of memory in the function of ion channels. They found that the longer the channel was held at a given voltage, the longer it took to recover from inactivation. The relationship between the duration of the holding voltage and the inactivation rate constants has a power-law form characteristic of a fractal scaling. Thus, the identification of short- or long-term memory in single-channel records may become an important tool when deciding about which model to apply in order to describe the experimental open- and closed-dwell time data.

The rescaled range analysis or R/S Hurst analysis is used in the present paper to determine if a single-ion channel has a fractal long-term memory. This analysis, in effect, compares correlation in the time series measured at different time scales. If the data are fractal, then the correlations at short time-scales are related to those at longer time-scales. A fractal long-term correlation found from the open and closed times of a channel suggests that the protein-forming channel has a different set of physical properties than those assumed by Markovian models. In a Markovian model, the kinetic rate constants are time independent and, physically, represent transitions between discrete conformational states of the channel protein that are separated by energy barriers. The fractal model assumes effective kinetic rate constants (K_{eff}) that depends on the time-scale at which the channel is recorded. Liebovitch *et al*. (1989) suggested that K_{eff} is a smooth and slowly varying function of time. This implies that the channel has a continuous sequence of nearly identical stable conformational states. One way to interpret such a model is that there are time-dependent dynamical motions within the three-dimensional channel structure produced by deterministic atomic, electrostatic, or hydrophobic forces.

In cellular biology, the rescaled range analysis has been used to analyse records in time produced by the mechanical motions of cells growing in tissue culture (Giaever & Keese, 1989) and one of us applied the R/S analysis to the study of patch clamp records of the membrane potential fluctuations of human T-lymphocytes (Churilla *et al*., 1996). Here the R/S analysis is applied to study calcium-activated potassium channels of Leydig cells. These cells are present in the testis and respond to the luteotropic hormone (LH) by increasing synthesis and secretion of testosterone in the male. A high conductance calcium-activated potassium channel was characterized in these cells with the patch clamp technique (Carnio & Varanda, 1995).

The Hurst method was also applied on computer-generated data using three- and four-state Markovian models for comparison with the experimental data.

Methods

EXPERIMENTAL TECHNIQUES

Cells

Leydig cells were freshly isolated from testes of Swiss mice weighing between 30–35 g as described elsewhere (Carnio & Varanda, 1995; Kawa, 1987). In short, the mice were killed by cervical dislocation, the testes surgically removed as rapidly as possible, decapsulated and injected

with Hank's balanced salt solution (HBSS). The injection procedure was repeated several times until the Leydig cells were washed away from the testicular mass. Once in suspension the cells were plated onto glass slides and allowed to adhere for 30–60 min, at room temperature (24–26°C).

Electrophysiology

The cells were transferred to a lucite chamber (0.3 ml) assembled onto the stage of an inverted microscope (Nikon-Diaphot TMD) and continuously perfused with any desired solution. Single-channel current events were recorded in the inside–out configuration of the patch clamp technique, as described in Hamill *et al.* (1981). Currents were measured with a List EPC-7 patch clamp amplifier (List Electronics), low passed at 2.5 kHz (8 pole-low-pass Bessel filter, Frequency Devices) and stored on the hard disk of an AT computer by sampling at 10 kHz with an A/D converter (Digidata 1200-Axon). Data acquisition and analysis were done with the PClamp suite of programs (PClamp6 Axon Instruments). Events with duration less than 100 μs were rejected and no correction was done for missed events. Micropipettes were pulled from the borosilicate glass capillaries (GSF-15 Clark Electromedical) with a puller (Narishige PP-83). They were Sylgard coated as close to the tip as possible and fire polished in a microforge (Narishige MF 83). Pipette resistances ranged from 10 to 12 MΩ when filled with any of the solutions used in the experiments. In this paper, the indicated voltages refer to the intracellular side. Therefore, outward currents are positive and represent the movement of positive charges from the cytoplasmic to the extracellular side of the membrane. All experiments ere carried out at room temperature (24–26°C).

Solutions

Cells were isolated and maintained in Hank's balanced salt solution with the following composition (mM): 145.0 NaCl; 4.6 KCl; 1.3 $MgCl_2$; 10.0 glucose; 1.6 $CaCl_2$; 10.0 HEPES; 5.0 $NaHCO_3$; pH 7.4. After getting the inside–out configuration the chamber was perfused with the following solution (mM): 150.0 KCl; 10.0 HEPES; 1.0 $MgCl_2$; 2.97 $CaCl_2$; 5 EGTA; pH 7.4, which was also present inside the pipette. Therefore, the single-channel recordings were made in the absence of ion gradients. The free calcium concentration was 10^{-7} M, as calculated using the program MaxChelator (Bers *et al.*, 1994). Solutions were made with doubly distilled water and filtered through 0.22 μm pore diameter filters (Millipore GSWP 02500).

THEORY

The R/S Analysis

The rescaled range analysis or R/S Hurst analysis is used to study records in time or a series of observations in different times (Bassingthwaighte *et al.*, 1994; Liebovitch, 1998). Hurst invented this new statistical method with the objective to study the flow of the Nile river and the problems related to water storage (Feder, 1988). The basic problem of Hurst was to determine the design of a reservoir based upon the given temporal record of the observed discharges from the lake. Here, the method of Hurst will be introduced considering a time-dependent function $F(t)$, where the time t assumes only integer values $1, 2, 3, \ldots, T$. The sum of the deviations of F in relation to the mean of the function $\{\langle F\rangle = 1/T\sum F(t),\ 1 \leqslant t \leqslant T\}$ allows us to define a new function as follows: $X(\tau, T) = \sum_\tau \{F(\tau) - \langle F\rangle\}$, in the range $1 \leqslant \tau \leqslant t$.

The above function represents the sum of the deviations from the mean at each point of the function from beginning of the time up to any instant t. This sum is repeated until t reaches the maximum value T.

Two other functions derived from $F(t)$ are defined. The first of them is the range R defined by $R(t) = \max X(t, T) - \min X(t, T)$, where $\max X$ and $\min X$ are the maximum and the minimum of $X(t, T)$ in the interval $1 \leqslant t \leqslant T$, respectively. The second function is the standard deviation $S(t) = \{(1/T)\sum[F(t) - \langle F\rangle]^2\}^{1/2}$, where the sum is over the discrete variable t, in the interval $1 \leqslant t \leqslant T$. Hurst found that the observed rescaled range, R/S (the range normalized by the standard deviation (S.D.)), for several types of temporal records is very well described by the following empirical relation: $R/S = (T/2)^H$. Hurst showed that the exponent H is more or less symmetrically

distributed about a mean of 0.73, with a standard deviation of about 0.09 in the different phenomena of nature. Thus, river discharges, rainfall, river and lake levels and so on, present a Hurst coefficient around 0.7. Feller (1951) showed that a sequence of mutually independent random variables with a common distribution is given by

$$E(R/S) = [\pi(T/2)]^H, \tag{1}$$

where $E(R/S)$ is the mean value of R/S after several executions of the experiment. A function $f(t)$ with this characteristic and $H = 0.5$ is said to be memoryless, since its present time value is independent of the past history of the function. When $H = 0.5$, the function $F(t)$ has memory. If $H > 0.5$ the function is called persistent because an increase in the present is more likely to be followed by a later increase. If $H < 0.5$ the function is antipersistent because an increase in the present is more likely to be followed by a later decrement.

In the case of single-channel events the time series consists of a recording of the ionic current during a certain time. Here, the function to be analysed is the time course or the activity of the single channel, sampled over N_t adjacent open and closed time intervals. The Hurst coefficients were calculated using the following strategy: (1) the data (adjacent open and closed time intervals) were divided into samples ranging in size from 2^1 up to a maximum of 2^{12} (2048 data); (2) the R/S mean value was calculated first by using the 1024 samples of 2^1 data, a second value of R/S was calculated using 512 sample of 2^2 data, and so on until a single sample of the total data (2^{12}) was reached; (3) the mean values of R/S were plotted against N_t (size of the samples) in a double-logarithmic plot; and (4) the value of the Hurst coefficient was then calculated from the slope of the straight line resulting from this type of plot.

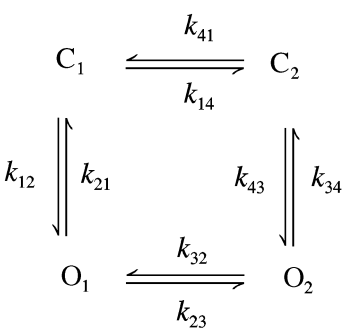
The ANOVA and Tukey tests were used to compare different Hurst coefficients. A Shapiro–Wilk's test was used to test whether the Hurst coefficients obtained from the simulated models are distributed according to a Gaussian distribution. The same test was used to test the normality of the H values of the shuffled data.

Models

The R/S analysis was first applied to data derived from the simulation of a linear three-state Markovian model, because this model describes the open- and closed-dwell time distribution of the experimental data very well.

The probability density function (p.d.f.) for the three-state Markovian model was obtained from the experimental data using the single-channel analysis program Pstat (Pclamp6—Axon Instruments). The rate constants were calculated as shown by Colquhoun & Hawkes (1981). The linear three-state Markovian model was simulated with the algorithm proposed by Liebovitch *et al.* (1987).

Despite the fact that a linear three-state model fits well the experimental data we also simulated a more general four-state model, which has been used to describe other large conductance calcium-activated potassium channels. This was done in order to test for the possibility that increasing the complexity of the model would produce autocorrelations that could be disclosed by the Hurst analysis. Therefore, a general four-state Markovian model in which two closed states (C_1 and C_2) make direct transitions to two open states (O_1 and O_2), through rate constants k_{ij}, was simulated according to the scheme below:



McManus *et al.* (1985) suggested that a model with these characteristics could generate an inverse relationship between lifetimes of adjacent open and closed intervals. Because solutions involving the Kolmogorov equations were very difficult, the four-state Markovian model was simulated using the following strategy: (1) the fraction of the open time was calculated using the open- and closed-dwell times from the experimental data; (2) k_{ij} values were chosen by trial such that the fractional open time of the

simulated four-state model was equal to that of the experimental data. The algorithm proposed by Liebovitch *et al.* (1987) was used to make the simulations.

To check that the programs were working properly we compared the simulated open- and closed-mean times (t_{mean}) with that theoretically expected and given by

$$t_{mean} = \frac{\int_0^\infty t f(t)\,dt}{\int_0^\infty f(t)\,dt},$$

where $f(t)$ is the probability density function (p.d.f.) of the simulated data, and t is the time.

A Macintosh microcomputer was used for the calculations and the programs were written in Microsoft BASIC.

Results

The sealing of a glass micropipette onto the surface of a Leydig cell results in the appearance of unitary current events in about 70% of the attempts. After confirming the presence of channels in the patch the micropipette was withdrawn from the cell and recordings were made thereafter in the inside-out configuration with a high potassium solution (150 mM KCl) present both in the bath and inside the pipette. Figure 1 shows traces of single-channel currents recorded at different holding potentials and with a fixed free-calcium concentration of 10^{-7} M. As can be seen the channels are voltage dependent: the probability of having an open channel (P_o) increases with depolarization.

The single-channel conductance, calculated from current amplitude histograms and I–V plots (not shown), is equal to 265 pS. Neither the conductance, nor the single channel kinetics are altered by filling the pipette with 140 mM potassium aspartate instead of KCl, suggesting that we are dealing with a K selective channel. These results characterize a calcium-activated potassium channel of large conductance as described elsewhere (Carnio & Varanda, 1995). Close inspection of the records in Fig. 1 shows that the time the channel spends in the open state is often followed by a much longer closed time interval. This pattern is clearly seen at 20 mV, but it remains even at a clamping voltage of 80 mV. This finding presents a qualitative behavior which could be seen in systems showing time correlation between the closed and open states.

Figure 2 shows distributions of the residence times in the open Fig. [2(a)] and closed states Fig. [2(b)] for a patch clamped at +40 mV. As can be seen the open time distribution can be adequately fitted by a single exponential function with a decay time constant equal to 0.56 ms. On the other hand, the closed time distribution requires at least two exponentials for a good fitting. In this case, the decay time constants are 0.17 and 86.38 ms for the fast and slow components, respectively.

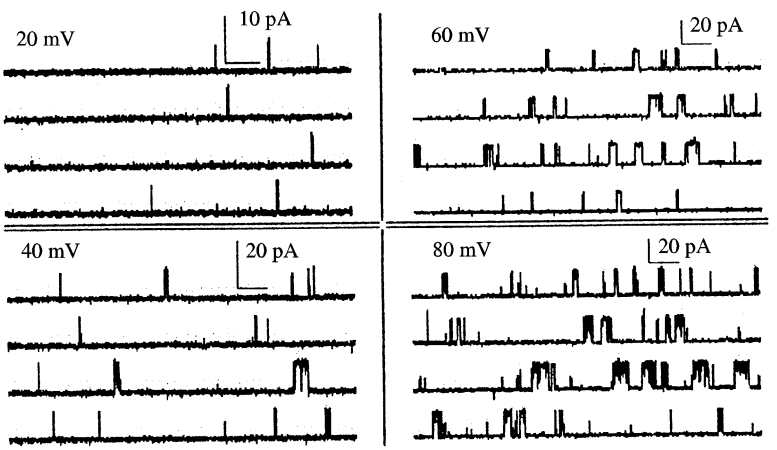


FIG. 1. Single-channel records of the calcium-activated potassium channel. The currents were recorded at the indicated voltages in the inside–out configuration. The solution of both pipette and bath were 150 mM KCl and the free calcium concentration was 10^{-7} M. Horizontal calibration bars indicate 50 ms.

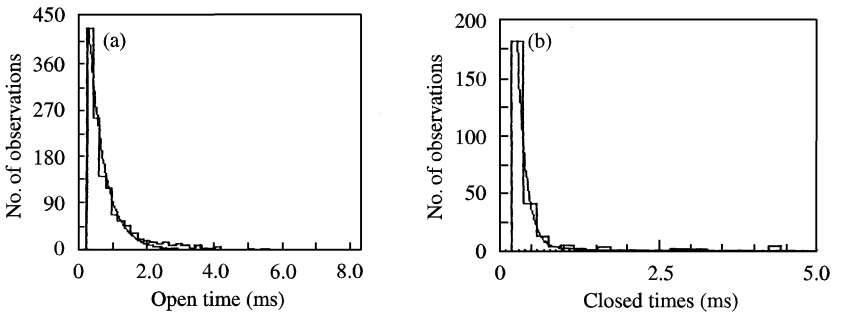


FIG. 2. Dwell time distributions to +40 mV for the channel shown in Fig. 1. (a) represents the open time and (b) the closed time distribution. The lines represent best fits of exponential functions to the experimental data points, using the Levenberg Marquardt algorithm. Number of events is 2330. Note that the histogram for the closed times has the abcissa expanded in order to better visualize the fitting. The exponentials are described in the text.

The p.d.f.'s that describe the open and closed time distributions were fitted by the following exponential functions: $f(t)_{open} = 1.776e^{-1.776t}$ and $f(t)_{closed} = 2.368e^{-5.848t} + 0.0069e^{-0.0116t}$ for +40 mV. These values were used to determine the rate constants of the three-state Markovian model that describes the kinetic of the calcium-activated potassium channel. The model can be represented as follows:

$$C_1 \underset{k_{12}}{\overset{k_{21}}{\leftrightharpoons}} C_2 \underset{k_{23}}{\overset{k_{32}}{\leftrightharpoons}} O.$$

Here C_1 and C_2 represent the closed states and O the open state of the channel and the K's are rate constants with the following values: $k_{12} = 28.50$, $k_{21} = 3455.8$, $k_{23} = 2375.3$ and $k_{32} = 1776.2$ Hz.

A channel clamped at +20 mV had its p.d.f. described by, $f(t)_{open} = 1.19\exp(-1.19t)$ and $f(t)_{closed} = 1.44\exp(-8.85t) + 0.00496\exp(-0.0059t)$ and the following k_{ij} values: $k_{12} = 1190$, $k_{21} = 1445$, $k_{23} = 7374.6$ and $k_{32} = 36.3$ Hz. For +60 mV the p.d.f.'s were $f(t)_{open} = 0.371\exp(-0.371t)$ and $f(t)_{closed} = 3.184\exp(-9.71t) + 0.035\exp(-0.053t)$ and k_{ij} values were $k_{12} = 371$, $k_{21} = 3220.1$, $k_{23} = 6385$ and $k_{32} = 157.98$ Hz. A channel clamped at +80 mV produced open- and closed-dwell time distributions des-cribed by the following p.d.f.'s: $f(t)_{open} = 0.87\exp(-0.87t)$ and $f(t)_{closed} = 3.127\exp(-6.369t) + 0.0319\exp(-0.063t)$. The k_{ij} values were $k_{12} = 870$, $k_{21} = 3159.1$, $k_{23} = 3146.2$ and $k_{32} = 126.7$ Hz.

Figure 3(a) shows the time series for a single-ion channel resulting from the simulations using a three-state Markovian model at +40 mV. The simulation used the same rate constants obtained from the kinetic analysis of the calcium-activated potassium channel. Although this three-state Markovian model can adequately describe the open- and closed-dwell time distributions of the experimental data, it produced significantly different values of the Hurst coefficients from those experimentally found. The H values resulting from simulations of the three-state Markovian models were 0.5498 ± 0.0180 ($n = 100$) for $V = +20$ mV; 0.5557 ± 0.0202 ($n = 100$) for $V = +40$ mV; 0.5565 ± 0.0246 ($n = 100$) for $V = +60$ mV and 0.5595 ± 0.0247 ($n = 100$) for $V = +80$ mV.

Figure 3(b) presents a double-logarithmic plot of R/S vs. the size of the sample (N_t) for a typical time series originated from simulations of the three-state Markovian model with the voltage clamped at +40 mV.

A four-state Markovian model was also simulated using rate constant values such that the fractional open time was equal to the value of the fractional open time of the experimental data. This strategy gave the following results for the kinetic rate constants: $k_{12} = 1700$, $k_{21} = 50$, $k_{23} = 10$, $k_{32} = 1500$, $k_{14} = 1000$, $k_{41} = 500$, $k_{34} = 50$ and $k_{43} = 1000$ Hz for a voltage clamped at +40 mV. For a voltage clamped at +80 mV the rate constant values were the following: $k_{12} = 1700$, $k_{21} = 10$, $k_{23} = 1000$, $k_{32} = 500$, $k_{14} = 200$, $k_{41} = 500$, $k_{34} = 10$ and $k_{43} = 1000$ Hz. For +20 mV the experimental

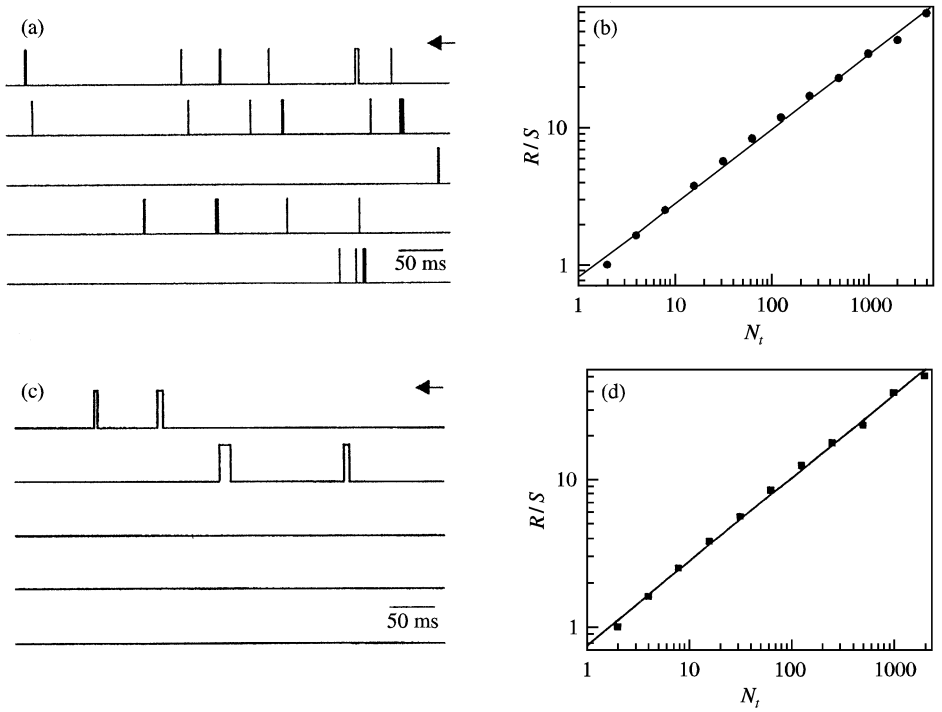


FIG. 3. R/S analysis applied to simulated single-channel data. The left panels (a) and (c) show three- and four-state Markovian models. Both simulations where done assuming the rate constants described in the text, at $+40$ mV. The right panels show double-logarithmic plots of R/S vs. N_t (size of the sample) for a typical simulation of the three [(b); $H = 0.534$; $R = 0.998$] and four-state [(d); $H = 0.557$; $R = 0.997$] Markovian models. Arrows indicate the open state of the channel. The continuous lines are best linear fits to the points and R is the correlation coefficient in each case.

fractional open time was practically equal to that found at $+40$ mV. The same occurred at $+60$ and $+80$ mV. Therefore, for this reason, the four-state Markovian model was not simulated at $+20$ mV and $+60$ mV.

Figure 3(c) shows the time series for a single-ion channel obtained from the simulations of the four-state Markovian model at $+40$ mV. Figure 3(d) is a double-logarithmic plot of R/S vs. the size of the sample (N_t) for a typical time series obtained from the above model. At this voltage the mean $\pm$S.D. value of H was 0.5554 ± 0.0171 ($n = 10$). For $+80$ mV the mean $\pm$S.D. value of H was 0.5508 ± 0.0333 ($n = 10$).

Figure 4 shows a double-logarithmic plot of the rescaled range R/S vs. the size of the samples (N_t) for a typical experimental record of the calcium-activated potassium channel voltage clamped at $+40$ mV, as shown in Fig. 1. The Hurst coefficient was obtained from the slope of the line fitted through the experimental points.

Values of H calculated for different voltages applied through the patch are shown in Table 1.

A Shapiro–Wilk's test showed that the values of H resulting from the three-state Markovian model simulated at $+20$ and $+80$ mV, are distributed according to a Gaussian function with the mean $\pm$S.D. given above for each voltage ($p_{Shapiro\text{–}Wilk's\ test} = 0.263472$ for $+20$ mV; $p_{Shapiro\text{–}Wilk's\ test} = 0.920086$ for $+80$ mV). These distributions were obtained by running 100 times the three-state Markovian model in each voltage. The chance that the value of a simulated H has to assume the same value as that of an experimental H in the same voltage is, in the most probable case, equal to 0.001. A z-distribution was used to determine this probability. At $+40$ and $+60$ mV the Shapiro–Wilk test shows that the data derived from the simulated three-state Markovian model do not conform very well with the Gaussian function ($p_{Shapiro\text{–}Wilk's\ test} = 0.040713$ for $+40$ mV; $p_{Shapiro\text{–}Wilk's\ test} = 0.048267$ for $+60$ mV). In these cases, the probability that

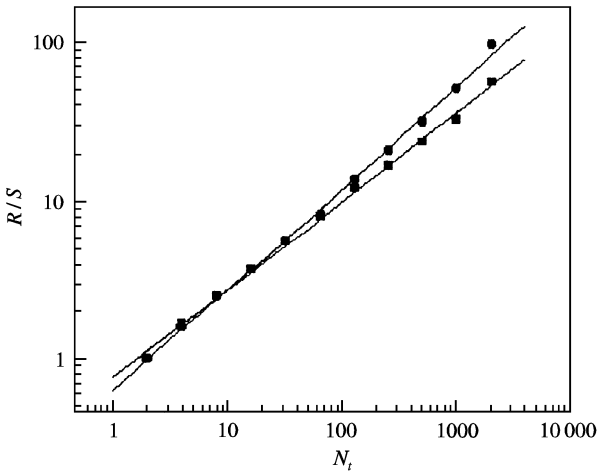


FIG. 4. R/S analysis for the channel shown in Fig. 1. R/S was calculated according to the text and plotted against N_t, the size of the sample. Data refer to the single-channel record observed at + 40 mV before (●; $H = 0.64$; $R = 0.998$) and after randomly shuffling the experimental dwell times (■; $H = 0.56$; $R = 0.998$). R is the correlation coefficient.

TABLE 1
Experimental values of H

Voltage (mV)	H (mean ± S.D.)	N
+ 20	0.634 ± 0.022	3
+ 40	0.635 ± 0.012	4
+ 60	0.606 ± 0.020	4
+ 80	0.608 ± 0.026	4

a simulated H has to assume the same or a higher value of an experimental H was calculated by counting the number of simulated H values equal or larger than the experimental H value, divided by the total number of H values simulated (100 values). In 100 simulations made at both voltages, this probability was equal to zero. Moreover, the ANOVA and Tukey tests showed that the experimentally determined H's are significantly different from that found for the four-state Markovian models ($F = 15.03550$; $p = 0.00001$). These results suggest the presence of openings and closings which are long-term correlated in time, i.e. there is memory in the kinetic process not disclosed by the theoretical models used. To further check this point we randomly shuffled the open- and closed-dwell times of the calcium-activated potassium channel voltage clamped at +40 mV, as shown in Fig. 1, and applied the R/S analysis to the resulting set of data. As expected, this process removed the correlation previously found in the experimental data ($H = 0.635$), giving now an H equal to 0.5689 ±0.0236 ($n = 100$; n represents the number of shuffling done on the experimental data file at +40 mV). The shuffled data were normally distributed ($p_{Shapiro\text{–}Wilk's\ test} = 0.906466$) and the chance that an H calculated from the shuffled data has to assume a value equal to that of the experimental Hurst coefficient is less than 0.002. A typical result of the randomly shuffled experimental data is shown in the plot of Fig. 4 (■).

Another interesting result is that the experimental values of H do not change with voltage, as shown by ANOVA at 5% significance level ($F = 2.2402$; $p = 0.140715$).

Discussion

The basic question involved in the study of time series resulting from a sequence of measurements of some quantity that fluctuates in time, is to find a statistical model that best describes its properties. In the kinetics of single-ion channel the time series is composed of a long sequence of opening and closing events characteristic of a particular process. The sequence of open and closed time intervals is considered to occur randomly as in a Poisson process. Therefore, the statistical distribution of the open and closed times should conform to an exponential p.d.f. This type of analysis allows the calculation of the rate constants defining the kinetics process by fitting one or more exponential functions to the open and closed time histograms. This classical Hodgkin–Huxley idea (Hodgkin & Huxley, 1952) that the rate constants are associated with a finite number of discrete states, which are related to different conformational states of the protein that forms the ion channel, is an assumption of all statistical Markovian analysis of single channels. In our case, the basic problem was to find out if there is long-term correlation between the open and closed time intervals without assuming that the kinetics of the channel follows a Poisson process. This aim was achieved with the use of the rescaled range analysis (R/S analysis). This non-classical statistical method was developed by

Hurst and was aimed at determining how the range of the fluctuations of the variable in study varies with the size of the sample previously chosen. When the range divided by the standard deviation (R/S), is proportional to $(N_t)^H$ where N_t is the size of the sample and $H > 0.5$, then the time series has a self-similar behavior. A Hurst coefficient around 0.6, as found in our experiments, indicates that there is open and closed time long-term correlation in the kinetics of this kind of channel.

A three- and a four-state Markovian models, which describe very well the open- and closed-dwell times distributions, were inefficient to describe the long-term correlation found between the open and closed states of this calcium-activated potassium channel. This fact indicates that models which assume that the channel-protein makes transitions only between discrete states of energy are not completely adequate to describe the kinetic process of single-ion channels, at least similar to the one shown here. Markovian models which assume the presence of more than one gate state may show memory and can generate correlations in the open and closed time records (McManus *et al*., 1985; Ball & Sansom, 1988; Colqhoun & Hawkes, 1987; Steinberg, 1987; Labarca *et al*., 1985; Jackson *et al*., 1983). The problem with them is that they are not able to describe fractal long-term correlation, as the Hurst analysis does. Our finding of these long-range correlations pertains to an important aspect of ion channel function, namely, the temporal pattern of openings and closings. However, this does not address all the issues of ion-channel function, such as the effects of ligand binding or modal gating. Recently, Kochetkov *et al*. (1999) have shown, using the Hurst analysis, that the behavior of single Ca^{2+}-activated potassium channels from cultured kidney *Vero* cells, is a multifractal process. The Hurst coefficients calculated by them suggest a slight positive correlation at short-time ranges ($H = 0.6$) and a strong one for long-time ranges ($N_t > 10^4$; $H = 0.88$). Despite this, they also simulated an eight states Markovian model, as used in McManus & Magleby (1991) and compared the results with the experimental data obtained from *Vero* cells. Again, as previously found for the simulated three states Markovian model (Nogueira *et al*., 1995), H was very close to 0.5, in clear contrast to the value of H obtained from their experimental data. In fact, Kochetkov *et al*. (1999) and another publication from our group (Nogueira *et al*., 1995) are the only reports published so far, showing that those correlations have self-similar fractal characteristics. As suggested by one of us (Liebovitch & Sullivan, 1987; Liebovitch, 1989; Liebovitch & Toth, 1990, 1991) a fractal model can account for this type of correlation. In that model, it is suggested that the channel exists in a large number of energy states, and the transitions among these states are determined by rate "constants" that change continuously in time.

The rescaled range analysis was also used by Churilla *et al*. (1996) to study the fluctuations of the membrane potential of human T-lymphocytes. They showed that the phenomenon has the fractal characteristics of a fractional Brownian motion. The calculated Hurst exponents were found to be in the range $0.78 \leqslant H \leqslant 0.80$. This high value of H for membrane potential records indicates a significant persistent correlation which may arise from nonlinear interactions between different time correlated single-ion channels causing long-term positive correlation in the macroscopic record of the membrane potential.

Another interesting point raised by our results is that the Hurst coefficient does not change with the voltage applied through the channel, at least for the sample sizes used in our experiments. Although the kinetics of the channel changes with depolarizing potentials, increasing the probability of finding it in the open state, the dynamics of the process remains unchanged, suggesting that memory is an intrinsic property of the system. This means that these fractal correlations arise from internal dynamics of the ion channel protein, which is quite distinct from saying that memory arises from a particular Markovian gate state. In other words, our results suggest that the energy barriers in the channel are changing in time and cannot be characterized by a Markovian model with a small number of fixed states.

The physical properties of ion channel proteins are not yet well known. However, it is likely that membrane proteins share many properties in common with globular proteins. The potential

energy function for globular proteins has a very large number of shallow, local minima (Karplus & McCammon, 1981; McCammon & Harvey, 1987; Welch, 1986). This is consistent with the fractal model and inconsistent with the few, deep minima of the Markov model. Experimental data and molecular dynamic simulations also show that the globular protein structures varies in time (Karplus & McCammon, 1981; Liebovitch, 1993) and this time dependence of the protein structure is consistent with the fractal model.

Finally, it is interesting to say that patch clamp data analysis of the distributions of the closed- and open-dwell times are insufficient to differentiate between Markov and fractal models. However, studies of the higher-order correlation properties of the data—such as the Hurst analysis carried out here—and molecular dynamics of the ion channel proteins should be the future direction of research that should permit to decide between Markov vs. fractal models.

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