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Fluctuation analysis of patch-clamp or whole-cell recordings containing many single channels ¹

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Fluctuation analysis of patch-clamp or whole-cell recordings containing many single channels¹

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Key words: Fluctuation analysis; Ion channel; Whole-cell current; Membrane noise; Digital signal processing

A novel analytical method for characterizing single-channel currents from recordings containing many identical, independent channels is described. The method is based on the assumption that the opening and closing of each single channel contributing to the summed current can be represented as a first-order, discrete-time, binary Markov chain and that the variance of the quiescent channel noise is known. Utilizing the first 3 moments of the record, and its power spectrum, all relevant single-channel parameters can be estimated. This includes the number of channels, the open current amplitude of a single channel, the mean open and closed durations and the probability of a channel being in the open state. In addition, the magnitude of the shot noise resulting from the flux of ions across the membrane can be estimated. Using fictitious multi-channel recordings generated by summing 2–990 independent binary Markov chains together with additive white noise, we have tested the reliability of the method in estimating the statistics of single channels. Finally, we discuss how the technique may be extended to cope with data which has been low-pass filtered, and also suggest further experiments which the technique now makes possible.

Introduction

It often happens that recorded transmembrane currents are not due to the activity of a single ion channel but arise from the collective behaviour of many channels that open and close intermittently. Such situations can occur when membrane currents are measured with intracellular electrodes, from whole-cell configurations or excised patches containing multiple channels. What information about the individual channels can be gleaned

from such current traces? In the case of identical channels that act independently, a solution to this problem was attempted by Conti and Wanke (1975). Modelling each channel as a binary Markov chain and working in continuous time, they obtained expressions for the mean, variance and power spectrum of the current record. Unfortunately, the model parameters themselves cannot be calculated from this information alone, unless the number of free parameters is reduced by imposing an unrealistic restriction on the transition probabilities. A method of ascertaining the rate constant for channel closing from the power spectrum of voltage-clamped intracellular recordings was devised by Anderson and Stevens (1973), who modelled the channel current as a Poisson process. Further work along these lines was presented by Siebenga et al. (1973).

By fitting a quadratic function to the mean-variance curve obtained from experiments at different levels of mean current, it is possible to

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estimate the number of channels and the open conductance level (Ehrenstein et al., 1970; Begelesich and Stevens, 1975; Sigworth, 1980; Holton and Hudspeth, 1986). Although this technique gives no information about the individual channel kinetics, it recognises the importance of solving the inverse problem, rather than simply providing expressions for the statistics of the process.

With the use of specific receptor agonists, pharmacological blocking agents and recombinant DNA techniques, it has now become possible to activate a single, homogeneous type of channel. This substantiates the assumption of identical channel current levels and kinetics and paves the way for a complete solution of the fluctuation analysis problem in the case of independent channels. We introduce a method that utilizes the mean, variance, third central moment and the power spectrum which enables a *complete identification* of the process from a whole-cell, intracellular or multi-channel patch recording containing additive white noise. Moreover, our method requires only *one current recording* to be taken at a *constant membrane potential*, thus facilitating the experimental technique.

By combining the various statistics, one can estimate the number of single channels contributing to the transmembrane currents, as well as the state amplitude (conductance level) and transition probabilities, which hitherto could only be revealed through single-channel recordings. We describe the method at an intuitive level, and refer the reader to Pulford et al. (1993) for rigorous derivations of the results we apply. We include here a practical guide for implementing the technique on a digital computer. Using simulated channel data, we demonstrate that the analytical scheme we propose can identify the process parameters with an acceptable degree of accuracy.

Overview of probabilistic channel models

We model a single channel's behaviour as a discrete-time stochastic process $\{x_k, k = 0, 1, 2, \dots\}$ in which x_k denotes the channel state which may be open or closed. We presuppose that the probability of the channel macromolecule assum-

ing the open or closed conformation depends only on its previous state, and that this probability is independent of the time. The single-channel process can therefore be represented by a first-order Markov chain with 2 states. We briefly compare and contrast 3 specific classes of discrete-time Markov processes which have been used in the past to model channel currents.

Bernoulli process

A simple way to envisage the Bernoulli process is a sequence of heads (H) and tails (T) obtained from tossing a coin, which may be either fair or biased. The process generated by a sequence of Bernoulli trials can be described by a single parameter p , the probability of observing a head given that a head was last thrown. Given that H was observed at trial 1, the probability of obtaining H again at trial 2 is p ; the transition probability from H to T from trials 1 to 2 is $1 - p$. Conversely, given that T was observed at trial 1, the probability of obtaining another T at trial 2 is $1 - p$, and the transition probability from T to H is p . Compactly, we can represent this stochastic process by a 2×2 transition probability matrix which reads:

$$A = \begin{pmatrix} p & 1 - p \\ p & 1 - p \end{pmatrix} \quad (1)$$

where the 2 entries in the first row represent the transition probabilities from H to H and H to T, whereas those of the second row are the transition probabilities from T to H and from T to T.

The Bernoulli process cannot adequately represent the kinetics of a single channel. This is because the power spectrum $S(\omega)$ obtained from such a process is always flat. Conversely, if the true process $S(\omega)$ has low-pass spectral characteristics, then it could not have been generated by Bernoulli trials. This assertion rests on the fact that the eigenvalues λ of the transition matrix given in Eqn. 1 are 0 and 1.

Discrete-time random telegraph signal

The so-called random telegraph signal, another 1-parameter stochastic process, exhibits different characteristics from the Bernoulli process.

Imagine that the telegraph key is in the open position at time t . The probability that the key will drop to the closed position at time $t + 1$ is p . Conversely, once the key is in the closed position, it will spring up to the open position with the same probability. The transition matrix of this process can be written as:

$$A = \begin{pmatrix} p & 1-p \\ 1-p & p \end{pmatrix} \quad (2)$$

One of the features of this process is that the mean closed and open durations are identical, and the proportion of time the process spends in one or the other state is precisely 0.5. In mathematical parlance, the eigenvector of the above matrix corresponding to the eigenvalue 1 is (0.5, 0.5). The power spectrum $S(\omega)$ of such a process has a low-pass characteristic, with a corner frequency which is related to p . To apply an analytical scheme based on this signal model, the experimental conditions, obtained for instance by adjusting the agonist concentration or temperature, would need to be contrived in such a way that channels remain open about 50% of the time during the experiment. The mean open time or rate constant for this model can only be derived from the power spectrum if this condition is satisfied.

General binary Markov chain

By introducing an additional parameter in the transition probability matrix, it is possible to generate a much greater variety of signal behaviours. The random process so generated is known as a Markov chain, which can be illustrated with a ball-tossing game. In this imaginary game, a ball is tossed back and forth between two players, called C and O. Player C tosses the ball to Player O with probability $1 - \zeta$, whereas Player O tosses the ball to Player C with the probability $1 - \rho$. The probability that Player C, being in possession of the ball, does not toss it to Player O is ζ . Similarly, the probability that O does not throw the ball to C is ρ . The transition probability matrix, for this example, is given by

$$A = \begin{pmatrix} \zeta & 1-\zeta \\ 1-\rho & \rho \end{pmatrix} \quad (3)$$

We note here that the random telegraph signal can be viewed as a special, restricted case of this Markov process corresponding to $\zeta = \rho$. The process generated by Eqn. 3 is known as a 2-state, first-order, homogeneous Markov chain.

Transition probability matrix and channel kinetics

Once ζ and ρ in Eqn. 3 are estimated, many of the statistics of the stochastic process can be deduced. The mean closed and open durations may be calculated as

$$\bar{d}_c = 1/1 - \zeta \quad (4)$$

$$\bar{d}_o = 1/1 - \rho \quad (5)$$

while the closed interval distribution, which has an exponential form, is given by

$$P_i(d) = \zeta^{(d-1)}(1 - \zeta). \quad (6)$$

where d is the interval in number of samples. The open interval distribution is obtained by replacing ζ by ρ . (For example, if ρ is estimated to be 0.96 and the sampling interval is 200 μ s, then the mean open duration is 25 points or 5 ms.) The proportion of time the process is in the closed and open states, known otherwise as the eigenvector (π_c, π_o) belonging to the unity eigenvalue, is related to ζ and ρ by

$$\pi_o = \frac{1 - \zeta}{2 - \rho - \zeta} \quad (7)$$

$$\pi_c = \frac{1 - \rho}{2 - \rho - \zeta}. \quad (8)$$

Finally, the eigenvalues λ of the transition matrix have a special significance in that, for a given set of state amplitudes, all the statistical properties of the Markov chain can be expressed using these quantities alone. For a 2×2 transition matrix, 1 of the 2 eigenvalues is always 1. The other can be computed as

$$\lambda = \zeta + \rho - 1. \quad (9)$$

The superposition of L identical channels will have $(L + 1)$ conductance levels with an $(L + 1) \times (L + 1)$ transition probability matrix. It can be shown that the $(L + 1)$ eigenvalues of such a matrix are

$$1, \lambda, \lambda^2, \dots, \lambda^L. \quad (10)$$

It is important to note that a given matrix uniquely determines its eigenvalues, but the matrix cannot be deduced from a set of eigenvalues alone.

Continuous-time vs. discrete-time representation

We can treat Markov processes in discrete or continuous time. If time is considered discrete, as arises in practice when we sample a continuous-time process, then the preceding discussions are applicable. When time is continuous, we can specify the Markov process in terms of its intensity matrix \mathbf{Q} . We can move between the two process descriptions by observing that the discrete-time transition matrix \mathbf{A} is the matrix exponential of \mathbf{Q} times the sampling period, namely, $\mathbf{A} = \exp(\mathbf{Q}T)$. In formulating the signal model, it makes little difference whether the process is envisaged as a continuous-time or discrete-time chain. However, the inverse problem of obtaining a model from the data is what we are faced with in practice. For any proposed model of channel dynamics, we need to obtain optimal estimates of the parameters featuring in it, and evaluate how well it describes the observation sequence.

The real world process or the measurement obtained in the laboratory invariably contains random noise in addition to the signal of interest. It is when dealing with these noisy observations that a continuous-time formulation of a Markov chain poses added technical difficulties. The mathematical tools for handling continuous-time processes, the realization of which is hidden in noise, are still being developed, and involve advanced mathematics such as Ito calculus and Wiener processes. The discrete-time formulation, on the other hand, avoids these theoretical problems. Moreover, a wide range of digital signal processing algorithms are already at our disposal.

Many identical channels

Once the transition probability matrix of the Markov process (and, for channel currents, the amplitude of the open state) is specified, the steady-state properties of such a chain are completely specified. These include: the mean open and closed durations, the distribution of open and closed times, the probability of being in the

open state, the power spectrum, and all statistical moments. No two realizations of a chain can be expected to be identical in the time domain but different realisations of the same stochastic process should be statistically indistinguishable from one another, given a sufficiently long segment of data. A corollary to this assertion is that information about the elements of the matrix, the amplitude of single-channel currents and the number of single channels contributing to the measured currents is able to be extracted from the statistical properties of the observation sequence.

Statement of the problem

Signal model

We make the following assumptions about the underlying signal sequence contained in multi-channel recordings. Firstly, the behaviour of a single channel can be approximated by a discrete-time, 2-state, homogeneous Markov chain. Secondly, each channel opens and closes independently of the others. Thirdly, all channels contributing to the record have nearly the same conductance levels and transition probability matrices.

The first assumption can be readily justified. Although it is of theoretical importance to ascertain whether channel kinetics are best represented by a Markov process or by some other stochastic process, the parameter estimation scheme is relatively robust to deviation from the first-order Markov assumption (Chung et al., 1990, 1991). The second assumption is likely to be valid. This is because a large number of ion channels distributed over a cell surface are likely to behave independently. Whether the independence assumption holds for adjacent channels contained in muscle endplates and excised patches of less than $1 \mu\text{m}^2$ can be answered experimentally. If the channels are not independent but partially coupled, the method we describe here may perhaps be extended to take this into account and obtain an estimate of this coupling parameter, but the associated mathematics would certainly be more complicated. Finally, the assumption of identicalness of the constituent channels must be

met by experimental manoeuvres. By using specific agonists or antagonists, recombinant DNA techniques or otherwise, the experimenter must ensure that the net current response stems from the activation of a population of homogeneous channels.

Formulation of the problem

Given observations of a process consisting of a sum of N identical, independent, 2-state channels, embedded in white gaussian noise, obtain the most likely estimates of the number N of single channels, the conductance level, the stationary probability distribution and the transition probability matrix.

Notation

We define the following notation and abbreviations which will be used frequently in the paper. We indicate matrices and vectors by bold type (see Table 1).

Estimation procedure

There are 4 unknown variables to be estimated: the number of channels N ; the 2 random variables ξ and ρ featuring in Eqn. 3; and the amplitude s of a single channel current. All other attributes of a channel's kinetics can be derived from these quantities. We therefore need 4 independent statistical measurements derived from the record. It is relatively easy to work with the mean, variance, third-order central moment and the power spectrum. These quantities are then expressed in terms of the unknown variables and the resulting equations solved to obtain estimates of the parameters.

Mean

Experimental data give measurements y_k that are the sum of the channel currents contributed by N independently and identically distributed (*iid*) 2-state Markov chains with additive, zero-mean white gaussian noise of variance σ^2 . We assume that the noise process n_k is independent of the signal process. We write the observable process as

$$y_k = \sum_{i=1}^N x_k^{(i)} + n_k \quad (11)$$

TABLE 1
NOTATION

x_k, y_k	scalar Markov chains
s	state (conductance) level
\mathbf{A}	transition probability matrix
N	number of channels
K	number of data points
t	time
k	discrete time index
T	sampling time
ξ	closed \rightarrow closed transition probability
ρ	open \rightarrow open transition probability
π_c	stationary probability of being in the closed state
π_o	stationary probability of being in the open state
HMM	Hidden Markov Model

where $x_k^{(i)}$ represent the *iid* binary Markov chains. Thus the mean value is

$$m_1(y) = N m_1(x^{(i)}) = N \pi_o s. \quad (12)$$

As no current flows when a channel is closed and the additive noise has zero-mean value, the terms π_c and σ^2 do not feature in the expression for the signal mean.

Variance

The second-order central moment, or variance, of the observable process is

$$\mu_2(y) = N \mu_2(x^{(i)}) + \sigma^2 = N \pi_o \pi_c s^2 + \sigma^2. \quad (13)$$

In passing, we note here that Eqns. 12 and 13 together give the following mean-variance relationship

$$\mu_2(y) = s m_1(y) - m_1^2(y) / N + \sigma^2. \quad (14)$$

This relationship holds for any binary Markov chain, including the Bernoulli process.

Third-order central moment

The third-order central moment of a process is related to the skewness of its probability distribution. In the case of a binary Markov chain, it is expressible as:

$$\mu_3(y) = N \mu_3(x^{(i)}) = N \pi_o \pi_c (\pi_c - \pi_o) s^3. \quad (15)$$

Note that the third central moment is independent of the noise variance.

Proceeding further, we can obtain the fourth- and higher-order central moments of the record,

but for binary Markov chains, the higher-order moments contain no additional information other than that which can be derived from the first 3 moments. A proof of this assertion is given in Fredkin and Rice (1986) for reversible, N -state, continuous-time Markov chains and Pulford et al. (1993) for discrete-time, binary Markov chains.

Power spectrum

The power spectrum of a discrete-time Markov chain is the Z -transform of the autocorrelation function evaluated on the unit circle, or

$$S_y(\omega) = NS_x(i)(\omega) + \sigma^2 \\ = \frac{N\pi_c\pi_o s^2(1-\lambda^2)}{1+\lambda^2-2\lambda \cos \omega T} + \sigma^2. \quad (16)$$

where λ is the non-unity eigenvalue of the transition matrix for a single chain.

The power spectrum of a discrete-time Markov chain or of the sum of N iid Markov chains shows a low-pass characteristic given by Eqn. 16. The spectrum is of course periodic, but is similar to a Lorentzian for low frequencies. By fitting the theoretical curve to the computed spectrum, we can estimate the eigenvalue λ , the net signal variance and the noise variance.

Identification of channel kinetics

By solving the 4 equations (Eqns. 12, 13, 15, and 16), we obtain expressions for N , π_c , and s . Recall that, by definition, $\pi_c + \pi_o = 1$. For notational convenience, we define a dimensionless variable γ as:

$$\gamma \triangleq \frac{m_1(y)\mu_3(y)}{(\mu_2(x))^2}. \quad (17)$$

Noting that $m_1(y) = m_1(x)$ and $\mu_3(y) = \mu_3(x)$ but $\mu_2(y) = \mu_2(x) + \sigma^2$ and $S_y(\omega) = S_x(\omega) + \sigma^2$, it follows that:

$$\pi_c = (2 - \gamma)^{-1} \quad (18)$$

$$s = \frac{\mu_2(x)}{m_1(y)\pi_c} \quad (19)$$

$$N = \frac{\pi_c m_1^2(y)}{\pi_o \mu_2(x)} \quad (20)$$

The diagonal entries of the transition probability matrix of a single channel are given by:

$$\zeta = \pi_c + \pi_o \lambda \quad (21)$$

$$\rho = \pi_o + \pi_c \lambda. \quad (22)$$

Estimation of shot noise

Shot noise results from the superposition of a large number of disturbances, such as the passage of charged ions across a membrane. The spectral characteristics generally depend on the origin of the disturbance. Rice (1944, 1945) uses the Fourier representation to describe the expected features of this noise, including the effects of non-linear filtering. For our purpose, it is sufficient to realize that the variance of random noise when channels are conducting will be larger than that obtained just before their activation. From the computed power spectrum, we can estimate the variance of noise σ^2 (see Eqn. 16), in addition to the non-unity eigenvalue λ of the transition matrix of a single chain. The difference between this estimated variance and the measured variance before the activation of channels can be viewed as the contribution due largely to shot noise.

Practical considerations

Several practical problems need to be considered before data acquisition and analysis can be carried out.

Zero-mean baseline

After the required transmembrane potential is applied, but before channels are activated, we need to obtain the noise variance from a control segment. It is assumed that the mean of the quiescent segment is zero, since by definition currents do not flow through a closed channel. If the control trace is displaced from the zero level owing to leakage currents or the presence of junction potentials, it should either be adjusted to give a zero-mean or its magnitude noted for subsequent subtraction from the experimental record to be analyzed.

Sampling frequency

To avoid aliasing when the continuous-time current signal is sampled, the sampling rate must be greater than or equal to the Nyquist frequency. Thus, if the record is filtered at, for example, 1 kHz, the sampling interval should be 500 μ s or higher. For a discussion of the sampling theorem, the reader is referred to Shannon and Weaver (1963) or Oppenheim and Schaffer (1975).

Data quantization

The output of the amplifier is usually quantized. If a 12-bit A-to-D converter is used for digitizing, care must be taken to ensure that the quantization levels are much smaller than the amplitude of the single channel currents. For example, if the record is digitized coarsely with a 5 pA resolution, the analysis method cannot be expected to identify correctly single-channel current amplitudes that are less than this value. To meet this requirement, the amplifier gain must be increased so that the record is commensurate with the allowed voltage range of the A-to-D converter. When there is a large displacement from the zero current level, owing to a large number of channels being activated, it may be necessary to re-zero the trace at the baseline by injecting a fixed opposing current. The exact amount of current injected must be taken into account in calculating the mean, the variance and the third central moment. AC-coupling the output to bring the displaced current trace near the baseline should not be attempted when using our technique. As an alternative we recommend the use of a 16-bit A-to-D converter.

Power spectrum and curve fitting

A method of power spectrum estimation that has been widely used is that of segmental averaging, also known as Bartlett's procedure (Welch, 1967; Jenkins and Watts, 1968). Although it has been criticized by some (e.g., Yuen, 1979), we find it is adequate for deriving the desired parameters.

Having obtained an estimate of the power spectrum of the data we must fit Eqn. 16 to this spectrum estimate. This is a non-standard curve-

fitting problem in that the fitting is in the log domain and we have logarithmically spaced measurements. A procedure suited to this task is a method based on a stochastic gradient descent algorithm.

Distortion of channel currents due to RC filtering

In a whole-cell recording mode, series resistance in conjunction with cell capacitance limits the bandwidth of the record to about 1 kHz. The bandwidth, however, can be slightly increased by making use of series-resistance and capacitance compensation controls of patch-clamp amplifiers. It is desirable to increase the bandwidth such that the effective filter formed by the electrode resistance and cell capacitance is greater than the filtering frequency imposed.

Filtering blurs sharp signal edges and causes attenuation of high frequency components present in the signal. To account for the effects of this form of distortion, we take the following approach. We assume that, for whole-cell voltage clamp measurements, the major band limiting effect is the capacitance of the cell and resistance of the electrode, which can be modelled as an RC filter, and that this time constant can be calculated a priori. We are therefore interested in adjusting the values we obtain for the statistics and power spectrum of the noisy signal to account for any filtering. The transfer function of a low-pass RC filter is

$$G(s) = \frac{1}{1 + sRC} \quad (23)$$

where RC is the filter time constant and s is the Laplace transform variable. We compute the zero-order hold discrete-time equivalent of this filter (Astrom and Wittenmark, 1984) as

$$H(z^{-1}) = \frac{(1 - \beta)z^{-1}}{1 - \beta z^{-1}}, \quad (24)$$

where $\beta = \exp[-T/RC]$ where T is the sampling time. Therefore, the input x_k and output y_k of the filter $H(z^{-1})$ are related by

$$y_k - \beta y_{k-1} = (1 - \beta)x_{k-1} + n_k \quad (25)$$

where n_k is the noise process. It is now a simple matter to calculate the statistics and power spec-

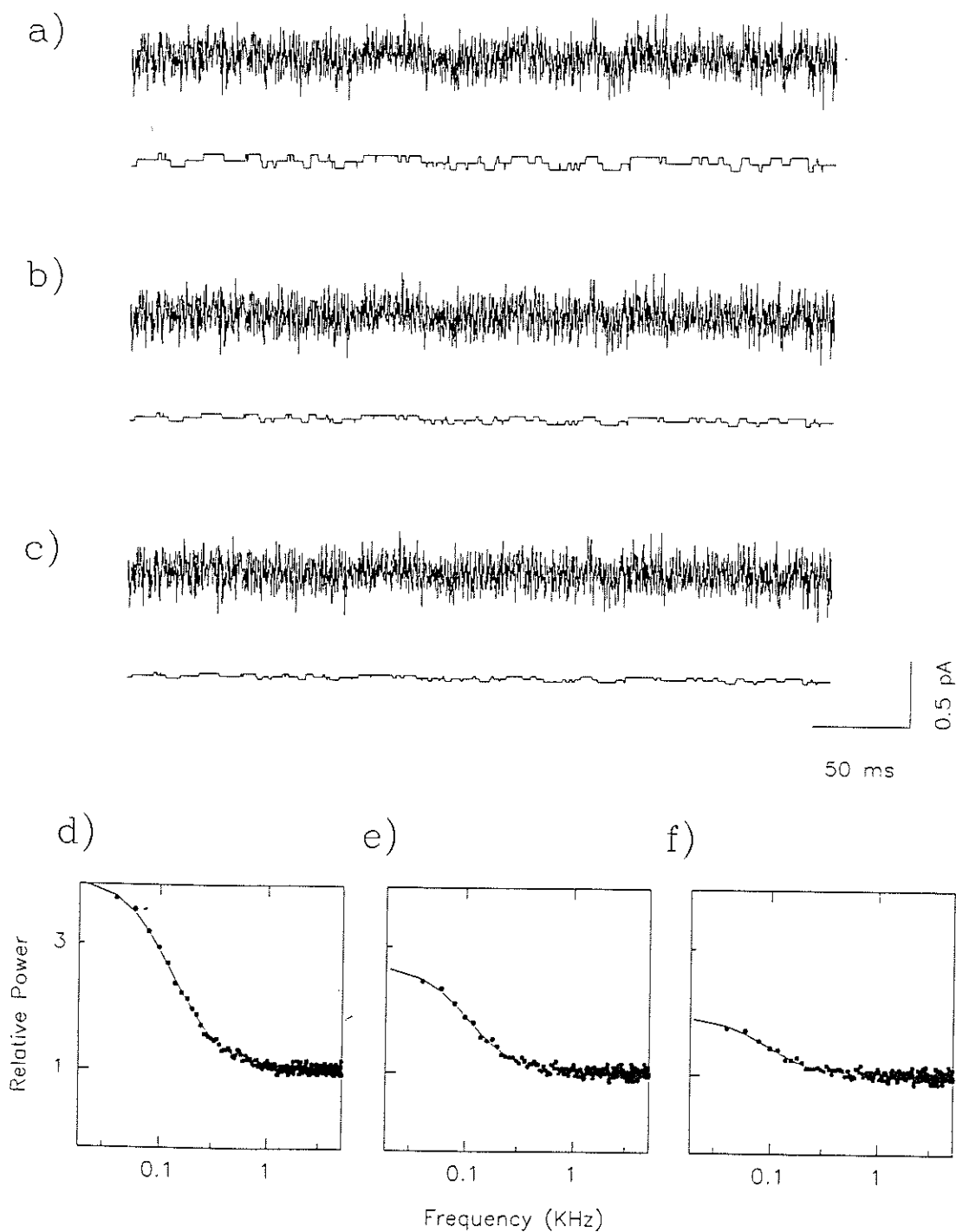


Fig. 1. Identification of individual channel kinetics from recordings containing 2 small single-channel currents. Traces of the first 1000 points are plotted for the noisy signal containing 2 single-channel currents with an amplitude of 0.05 pA and the corresponding signal sequence in the absence of noise (a). The standard deviation of the noise was 0.1 pA. The amplitude of the signal was reduced to 0.03 pA (b) and then to 0.02 pA (c). The power spectra obtained from the data illustrated in (a), (b) and (c) are shown in (d), (e) and (f). The solid lines represent fitted curves using Eqn. 16.

trum of the underlying Markov signal x_k from the noisy, filtered measurements y_k . We provide in the Appendix the necessary details for these computations, assuming that the noise is white and uncorrelated with the signal process.

Validation of the method

Data generation

A signal sequence composed of a prespecified number of identical Markov chains was first generated and then zero-mean gaussian noise was added. The amplitude of each chain and the length of the data were varied systematically. To mimic real data, we assume that, for excised patches, the record is filtered at 2 kHz (digitized at 5 kHz) and the standard deviation of the noise is 0.1 pA. For whole-cell recordings, we assume the cut-off filter frequency and the noise standard deviation are, respectively, 1 kHz (digitized at 2 kHz) and 1 pA. Throughout we express the amplitude of the signal in pA (or fA) and the time in ms. These units are arbitrary as far as the simulations are concerned and have only been adopted in keeping with experimental data.

Computation of the moments

The mean and variance were calculated using standard formulae. These 2 moments can be estimated with a relatively high degree of accuracy by treating all data points as a single array. The estimate of the third central moment sometimes deviated considerably from its true value, especially when the number of constituent chains was 100 or more. This is due to the presence of outliers in the simulated data which have a large effect on the skewness of the probability distribution. In practice, the data should be scanned for

such artifacts and some criterion for their elimination should be adopted, as the information contained in the tails of the distribution tends to be unreliable. An alternative to a direct computation of the third-order central moment is the fitting of a representative function to the amplitude histogram (probability density estimate) of the data from which this statistic could be computed analytically.

Power spectrum

Power spectra were calculated as the segmental average of the magnitude of the fast Fourier transform of non-overlapping blocks of data. A block length of 512 points was used and a Hanning window was applied to the data block prior to transforming. The power spectrum estimate was then normalised so that the signal energies in the time and frequency domain were equal. A smooth curve was fitted to the resulting power spectrum using a stochastic gradient descent algorithm.

Effects of signal-to-noise ratio

We illustrate the procedures involved in the parameter estimation with a segment of fictitious channel current in which 2 pores open and close independently. Although the entire estimation scheme has been integrated in a single program, we decompose the procedures into 3 separate computational steps for illustrative purposes.

Fig. 1a shows four 1000-point segments of the observation sequence, in which 2 single channels were activated. The standard deviation of the noise was set at 0.1 pA (100 fA), and the amplitude of the signal was reduced progressively from 50 fA (a) to 30 fA (b) and then to 20 fA (c). The

TABLE II
TRUE AND COMPUTED STATISTICS FOR THE 3 DATA SETS

Data	Mean (pA)	Variance (pA ²)	Third central moment (pA ³)
(a)	$-3.99 (-4.00) \times 10^{-2}$	$112.2 (112.0) \times 10^{-4}$	$-11.95 (-12.0) \times 10^{-6}$
(b)	$-2.39 (-2.40) \times 10^{-2}$	$104.6 (104.3) \times 10^{-4}$	$-3.12 (-2.56) \times 10^{-6}$
(c)	$-1.59 (-1.60) \times 10^{-2}$	$102.2 (101.9) \times 10^{-4}$	$-1.53 (-0.77) \times 10^{-6}$

transition probabilities used to generate the signal sequence were $\zeta = 0.98$ and $\rho = 0.97$. These correspond to a mean closed and open duration of 10 ms and 6.6 ms, respectively, for each channel. The probability of each channel being in the open state was 0.4. The analysis files consisted of 500,000 points.

The first estimation step is to calculate the moments of the record. For the 3 segments illustrated, the estimated mean in pA, variance in pA^2 and third central moment in pA^3 are displayed in Table II with the true values given in brackets. After computing the fitted power spectrum of the noisy data using the method described previously, estimates of the non-unity eigenvalue, as well as the signal and noise variances can be obtained.

The power spectra calculated from the 3 records are shown as filled circles in Fig. 1d-f, together with the fitted curves (solid lines). The

TABLE III

PARAMETER ESTIMATES FOR THE 3 DATA SEGMENTS

s	\hat{s}	$\hat{N}(2)$	$\hat{\zeta}(0.98)$	$\hat{\rho}(0.97)$
-50 fA	-51.5	2	0.977	0.969
-30 fA	-31.7	2	0.980	0.969
-20 fA	-21.2	2	0.983	0.967

estimated eigenvalues for (d), (e) and (f) were, respectively, 0.9472, 0.9492 and 0.9496, compared to the true value of 0.95.

The final step of the estimation procedure entails finding the 4 signal parameters N , s , p and ζ , using the relationships given previously. The estimated variables (denoted by a circumflex) together with the actual values (in parentheses) used in the simulation are given in Table III.

The number of single channels was correctly identified as 2 for all data files, and the estimates

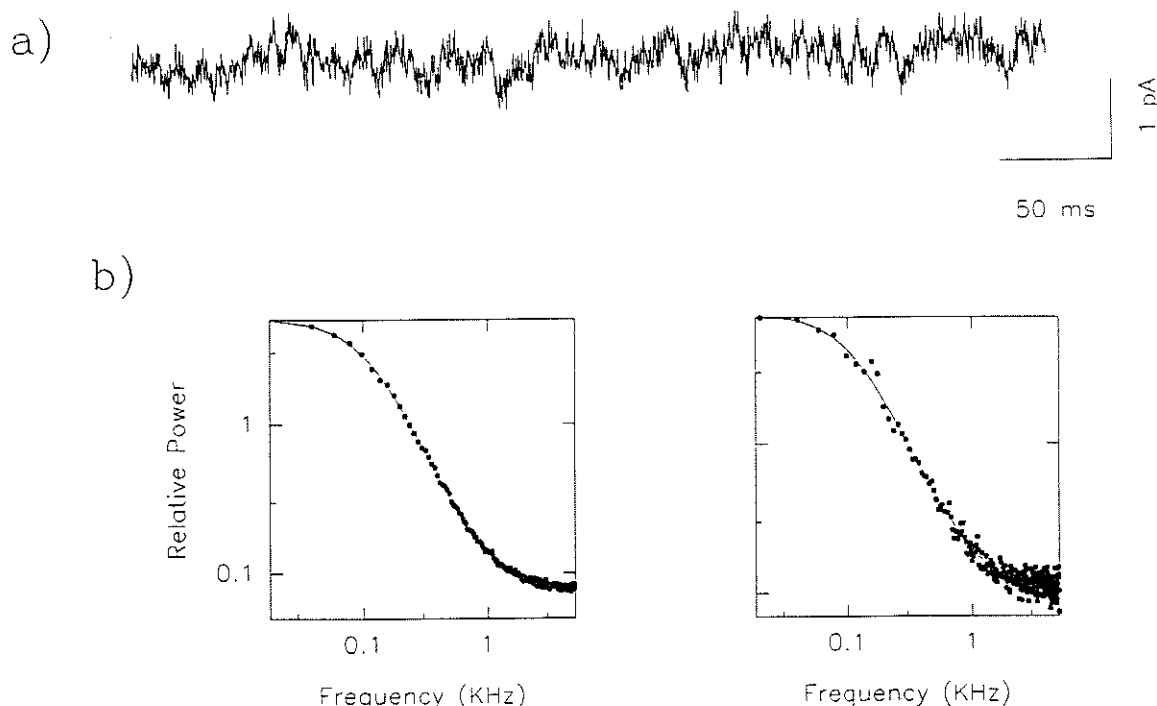


Fig. 2. Increase in estimation errors with decreasing data length. A segment of 1000 data points shown in (a) is composed of the summed currents contributed by 10 single channels. The amplitude of each single channel was 0.1 pA, as was the standard deviation of the noise. The power spectrum shown in (b) were estimated from a file that contained 500,000 points, whereas that shown in (c) were estimated from 10,000 data points.

of the transition probabilities were very close to their true values. The estimated current amplitude of a single channel deviated from the true value by 6% (21.2 fA as opposed to 20.0 fA) when the true signal amplitude was one-fifth the standard deviation of the noise.

Data length and estimation errors

It is desirable to ascertain how much data is required to obtain reliable estimates of the parameters. The minimum length of data needed for obtaining estimates with an acceptable degree of accuracy depends on several factors, such as the signal-to-noise ratio, the number of pores and the probability of opening. We show here that accurate estimates of the parameters can be obtained with a record containing 25,000 data points. In Fig. 2a, a short segment of simulation data is shown. The record contains 10 single channels, each with an amplitude of 0.1 pA, and the standard deviation of the noise in which the signal is embedded is 0.1 pA. The transition probabilities are $\zeta = 0.97$ and $\rho = 0.96$.

The data length used in the parameter estimation was systematically reduced from 500,000 points to 10,000 points. As shown in Fig. 2b,c, the quality of the power spectrum estimate degrades as the data length decreases. However, the estimated eigenvalue, along with the other estimated statistics, did not deviate appreciably from their true values until the length of the data was less than 25,000 points. The estimates obtained from a 10,000 point segment of data contained unacceptably large errors, suggesting that at least 25,000 points (or a real time record lasting 5 s)

TABLE IV
PARAMETER ESTIMATES FOR VARIOUS DATA SEGMENT LENGTHS

Length	\hat{N} (10)	\hat{s} (-100)	$\hat{\zeta}$ (0.97)	$\hat{\rho}$ (0.96)	$\hat{\pi}_o$ (0.429)
500,000	10	-103 fA	0.970	0.962	0.438
200,000	10	-105 fA	0.970	0.961	0.441
50,000	10	-103 fA	0.972	0.964	0.434
25,000	10	-103 fA	0.972	0.960	0.409
10,000	11	-98 fA	0.972	0.957	0.392

TABLE V

BEHAVIOUR OF PARAMETER ESTIMATES WITH VARYING ζ

ζ	\hat{N} (50)	\hat{s} (-1.0)	$\hat{\zeta}$	$\hat{\rho}$ (0.98)	$\hat{\pi}_o$
0.99	56	-0.94	0.990	0.978	0.313 (0.333)
0.98	53	-0.96	0.980	0.979	0.494 (0.500)
0.97	47	-1.03	0.967	0.980	0.620 (0.600)
0.96	48	-1.03	0.958	0.981	0.682 (0.667)
0.95	42	-1.13	0.947	0.982	0.749 (0.714)

are needed for the analytical technique described here. In Table IV, the estimates of the parameters obtained using various data segment lengths are listed.

Although, in this example, increasing the data length beyond 25,000 points results in only a marginal improvement in estimation accuracy, we take the data length as 500,000 points in subsequent examples to allow for cases involving large numbers of pores.

Frequency of channel opening

We generated a series of signal sequences containing 50 single channels, each with an open current level of 1 pA and $\rho = 0.98$. Assuming that such records in experimental situations are obtained from whole-cell configurations, we added gaussian noise with a standard deviation of 1 pA to the signal sequence.

While maintaining a data file length of 500,000 points and keeping $\rho = 0.98$, the transition probability ζ was systematically varied from 0.99 to 0.95. The results of the simulations showed that the parameter estimates begin to drift from the true values when constituent single channels remain predominantly in the open or closed state, as illustrated in Table V.

Large numbers of channels

We generated several multi-pore signals containing a large number of single channels, each with an open-channel current level of 1 pA, $\zeta = 0.98$ and $\rho = 0.97$. The number N of single chan-

TABLE VI
BEHAVIOUR OF PARAMETER ESTIMATES WITH
VARYING NUMBERS OF PORES

N	\hat{N}	$\hat{s} (-1.0)$	$\hat{\zeta} (0.98)$	$\hat{\rho} (0.97)$	$\hat{\pi}_o (0.4)$
250	242	-0.943	0.977	0.966	0.403
500	479	-0.966	0.978	0.972	0.432
750	721	-1.020	0.980	0.970	0.407
990	937	-1.030	0.979	0.971	0.410

nels contributing to the total current was systematically increased, keeping all other parameters constant. The signal sequences were contaminated by additive white noise with a standard deviation of 1 pA.

In general, the estimated number of single channels tended to be less than the correct number. The estimated transition probabilities and the amplitude, on the other hand, were all within 5% of their true values. In Table VI, the results of one such series of simulations are displayed.

Partially coupled Markov chains

Although the analytical method is based on the assumption that all channels contributing to the summed current open and close independently, we show here that the parameter estimates are still acceptably accurate even when the pores are weakly coupled. When the channels are strongly coupled, opening and closing nearly in synchrony, our technique erroneously identifies the underlying signal sequence as comprising a single channel of large amplitude.

There are a number of reports claiming that multiple channels contained in an excised membrane patch are not totally independent but partially coupled (Yeramian et al., 1986; Queyroy and Verdeti, 1992). We have devised a mathematical scheme for representing the behaviour of such coupled channels. With no loss of generality, we suppose that there are 2 identical channels with transition probability matrix \mathbf{A} . If the channels are totally independent, one will observe a 3-state signal sequence the 3×3 transition matrix \mathbf{A}_I of which is given by:

$$\mathbf{A}_I = \mathbf{L}(\mathbf{A} \otimes \mathbf{A})\mathbf{R} \quad (26)$$

where \otimes denotes the tensor product and \mathbf{L} and \mathbf{R} are aggregation matrices. On the other hand, the transition matrix \mathbf{A}_C of a pair of totally coupled channels can be written as:

$$\mathbf{A}_C = \begin{pmatrix} \zeta & 0 & 1 - \zeta \\ 1 - \delta & 0 & \delta \\ 1 - \rho & 0 & \rho \end{pmatrix} \quad (27)$$

where ζ and ρ have the same significance as in Eqn. 3. The bias probability δ determines the transition tendency of initially open or closed states toward the fully open or fully closed state. When the constituent channels are neither fully coupled nor totally independent, the matrix of such a partially coupled Markov chain can be defined as:

$$\mathbf{A}_{PC} \triangleq \kappa \mathbf{A}_C + (1 - \kappa) \mathbf{A}_I, \quad 0 \leq \kappa \leq 1. \quad (28)$$

where κ is the coupling factor. Although, for simplicity, we have illustrated the coupling scheme for 2 binary chains, it can readily be generalized to the case of L coupled chains.

Using the above scheme, we generated signals composed of 4 individual channels with various coupling strengths. In Fig. 3, the gating behaviour of 4 partially coupled channels is illustrated. When the coupling coefficient is small but non-zero ($\kappa = 0.025$), the channels open and close essentially independently but occasionally some or all 4 channels transit from the partially or fully open states to the closed state together (Fig. 3a). When the coupling strength is increased further ($\kappa = 0.10$), the channels open and close together more frequently, but the current still dwells at intermediate levels (Fig. 3b). For values of κ greater than about 0.20, the summed currents appear as a single channel, except that there are numerous brief flickers and some longer sojourns to submaximal current levels (Fig. 3c).

The results of our simulation, tabulated in Table VII, indicate that the departure from the independence assumption does not appreciably increase the estimation error, provided that the channels are weakly coupled. When the coupling is so strong that all 4 channels act as 1 group, our analysis method identifies the number of pores as 1, with the estimated amplitude being close to the summed amplitude. For intermediate values of κ ,

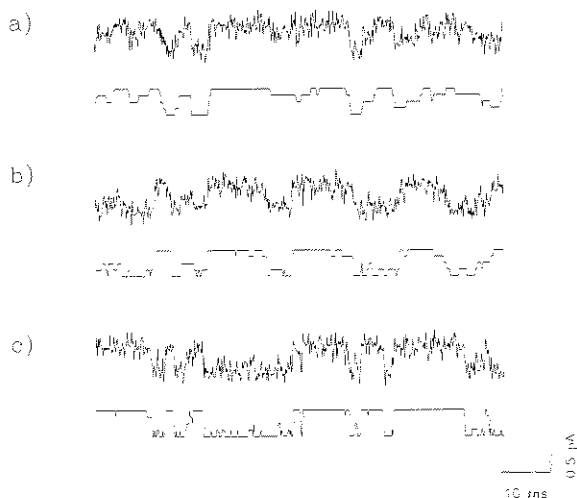


Fig. 3. Partially coupled single channels. Four identical single channels were partially coupled by using 3 different values of the coupling coefficients, κ . Segments of 500-point records of the noisy signal and pure signal shown in (a), (b) and (c) were obtained with $\kappa = 0.025$, $\kappa = 0.10$ and $\kappa = 0.4$, respectively. The amplitude of each single channel and the standard deviation of the noise were kept at 0.1 pA throughout.

the estimated number of channels was 2, instead of the true value of 4. Because 2 partially dependent channels appeared as 1 channel, the estimated probability of being in the open state and the mean open duration were about twice those of uncoupled single channels. We emphasize that the value of the coupling factor κ cannot be estimated using the present scheme. A procedure for identifying all the parameters of a coupled Markov chain is described elsewhere (Kennedy and Chung, 1992).

TABLE VII
ESTIMATION OF PARTIALLY COUPLED CHANNEL PARAMETERS

κ	$\hat{N} = 4$	$\hat{s} (-0.1)$	$\hat{\xi} (0.98)$	$\hat{\rho} (0.95)$	$\hat{\pi}_o (0.286)$
0.000	4	-0.097	0.981	0.949	0.269
0.025	4	-0.136	0.983	0.948	0.250
0.100	2	-0.242	0.964	0.966	0.518
0.400	1	-0.356	0.965	0.965	0.506

Aggregated Markov chains

The analysis of recorded single-channel currents reveals that the binary signal sequence can best be approximated by an aggregated first-order Markov chain in that the closed state may represent 2 (or sometimes more) kinetically distinct states. If the underlying chain has 3 states, the transition probability matrix \mathbf{A}_A for the discrete-time chain can be written as:

$$\mathbf{A}_A = \begin{pmatrix} p_{11} & p_{12} & 0 \\ p_{21} & p_{22} & p_{23} \\ 0 & p_{32} & p_{33} \end{pmatrix} \quad (29)$$

The observable current process is obtained by aggregating states 1 and 2 in the above model as follows. When the process is in state 1 or state 2, the channel remains closed, but it opens if the third state is reached. Technically, this aggregated binary process is not a Markov chain, and thus our analytical scheme cannot be applied. The matrix given in Eqn. 29 approximates a process in which the closed state represents an aggregate of 2 kinetically distinct conformational states (Colquhoun and Hawkes, 1982), although discretization of a continuous-time Colquhoun-Hawkes model will not give rise to zeros in the transition matrix. However, the following simulation results serve to illustrate that we can still obtain useful estimates of an equivalent 2-state Markov model, even if the true model is non-Markovian.

The transition probabilities of \mathbf{A}_A were taken to be: $p_{11} = 0.98$, $p_{21} = 0.01$, $p_{22} = 0.97$, $p_{23} = 0.02$, and $p_{33} = 0.97$. The amplitude of each channel was 0.1 pA, as was the standard deviation of the noise. We generated 2 sets of data using this channel model: one for 2 channels and the other for 10 identical channels. In both cases the algorithm correctly identified the number of channels. The estimated amplitudes of single channels were -0.097 pA and -0.101 pA for the 2 chains, compared with the true value of -0.1 pA. The estimated mean open durations were 6.45 ms ($\rho = 0.9690$) for the chain containing 2 channels and 6.65 ms ($\rho = 0.9699$) for the second chain. The estimated mean closed duration for each chain was 15.4 ms. The measured mean

closed duration of the signal sequence generated by Eqn. 29 was 15.1 ms.

Discussion

The method presented here for the analysis of current fluctuations is capable of yielding all the desired information about the gating behaviour of single channels. This includes the number of channels, the elementary conductance level and the transition probability matrix. Once these quantities have been identified, all other statistics of the single channel can be computed. There are several advantages in studying a population of channels activated simultaneously under the same experimental conditions. The kinetic constants estimated in this way represent an average value of many channels, which can only be obtained by averaging numerous measurements of single-channel recordings. In addition, the way in which the number of activated channels changes under certain experimental manoeuvres may also provide useful information about the dynamics of channel gating. Above all, there will be less need to resort to single-channel recordings, which is a technically demanding and labour-intensive task.

It is worth reiterating the assumptions we make about the underlying random processes contributing to the record. Our analytical method rests on the following 2 premises: (1) the signal sequence of each single channel can be represented by a binary Markov chain, and (2) all N channels have similar conductance levels and transition matrices. The processing scheme is relatively insensitive to the deviation from first assumption (see, e.g., Chung et al., 1990, 1991). Guaranteeing that the second assumption holds, which also applies to single-channel recordings, rests on the skill of the experimenter. With specific receptor agonists and antagonists as well as channel blockers, or by utilizing recombinant DNA techniques, we believe this condition can be easily met.

Using simulated signal sequences, we have assessed the reliability and limitations of the fluctuation analysis method. We showed that multiple independent channels can be characterized accurately, even when the amplitude of the individual

channel current is very small (Fig. 1) and the available observation sequence is short (Fig. 2). Currents recorded with a whole-cell configuration are likely to be due to the activity of many hundreds of single channels. The results of our simulation studies demonstrate that the dynamical parameters of the individual channel currents can be deduced with a fair degree of confidence when the modelling assumptions are valid. The accuracy of the parameter estimator was relatively insensitive to departures from these assumptions. Thus, our analytical method can effectively be applied to a collection of channels that are not totally independent (Fig. 3), or when the channels obey a Colquhoun-Hawkes gating model. The method, however, does not reveal if channels are independent nor, in the latter case, can it specify how many hidden states are aggregated into one observable state.

Utilizing the power spectrum and other statistical measures to deduce the kinetics of elementary processes is a well-known technique that has already been fruitfully exploited (e.g., Anderson and Stevens, 1973; Siebenga et al., 1973). It is important to realize that the corner frequency of the power spectrum is related to the non-unity eigenvalue λ of the transition probability matrix, not the mean open time (see Eqn. 16). For a binary process, λ is related to the elements of the transition matrix by Eqn. 9, namely, $\lambda = \zeta + \rho - 1$. Thus, to infer the mean open duration from the corner frequency of the power spectrum, it has to be assumed that either $\zeta = \rho$ (i.e., the process is a random telegraph signal), or $\zeta \gg \rho$ and $\zeta \approx 1$ (i.e., the mean closed duration is far greater than the mean open duration and channels seldom open). Although rarely stated explicitly, the estimates of channel kinetics based on fluctuation analysis in the past rest on these assumptions. It is also important to appreciate that 4 unknown parameters, namely the number of channels, the conductance level and ρ and ζ of the transition matrix, cannot be estimated from 3 independent measurements alone, for example, the mean, variance and the power spectrum. Either one has to reduce the number of parameters by 1 by making an unrealistic assumption about the underlying stochastic process or utilize an additional

measurement that is independent of the 3 listed above. Our method allows a general 2-state Markov channel model to be adopted, and utilizes the third central moment to estimate all 4 parameters.

The identification method we have described here may prove to be a useful tool for investigating certain properties of ion channels in living membranes. One potential application of the technique that we envisage is the quantitative determination of the thermodynamical properties of channel macromolecules. The changes in the model parameters with, for example, temperature or agonist concentration will provide good numerical estimates of the activation energy, the potential energy barrier, the enthalpy of the system, as well as the amount of energy donated by an agonist molecule to the receptor-channel complex. Also, the changes in the gating behaviour of a specific receptor population before and after a pathway is tetanized can readily be ascertained using our method. The fluctuation analysis technique can also be applied to cell membranes for which single-channel recordings are particularly difficult to carry out.

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APPENDIX

Statistics of RC-filtered Markov process

Mean. Taking the expectation of (25), and noting that $E\{n_k\} = 0$, it follows that

$$\mu_y = (1 - \beta)\mu_x / (1 - \beta) = \mu_x \quad (30)$$

where μ_x and μ_y denote, respectively, the mean of the original and RC-filtered signals, so that

there is no correction for the signal mean due to filtering.

Second moment. Squaring (25) then taking expectations yields

$$\begin{aligned} (1 + \beta^2)E\{y_k^2\} - 2\beta E\{y_k y_{k-1}\} \\ = (1 - \beta)^2 E\{x_k^2\} + \sigma^2 \end{aligned} \quad (31)$$

where σ^2 is the noise variance which we assume to be known, it now follows that

$$E\{x_k^2\} = \frac{(1 + \beta^2)E\{y_k^2\} - 2\beta R_{yy}[1] - \sigma^2}{(1 - \beta)^2} \quad (32)$$

in which we have used $R_{yy}[1] = E\{y_k y_{k-1}\}$ to denote the autocorrelation of y_k at a lag of 1 sample.

Third moment. Taking the cube of (25) and computing expectations gives

$$\begin{aligned} (1 - \beta^3)E\{y_k^3\} - 3\beta E\{y_k^2 y_{k-1}\} + 3\beta E\{y_k y_{k-1}^2\} \\ = (1 - \beta)^3 E\{x_k^3\} + 3(1 - \beta)E\{x_k\}\sigma^2 \end{aligned} \quad (33)$$

Defining $C_y[k, l] = E\{y_p y_{p-k} y_{p-l}\}$ results in a formula for the third moment of x_k :

$$\begin{aligned} E\{x_k^3\} = [(1 - \beta^3)E\{y_k^3\} + 3\beta(\beta C_y[1, 1] \\ - C_y[0, 1]) - 3(1 - \beta)\sigma^2 \mu_x] \\ \times [(1 - \beta)^3]^{-1} \end{aligned} \quad (34)$$

Central moments. Once the above quantities have been estimated from the filtered data, it is straightforward to compute the second and third central moments of the signal x_k , for instance, we obtain for $Var(x)$

$$\begin{aligned} Var(x) \\ = E\{x_k^2\} - (E\{x_k\})^2 \\ = \frac{(1 + \beta^2)\mu_y - 2\beta R_{yy}[1] - (1 - \beta^2)\mu_y^2 - \sigma^2}{(1 - \beta)^2} \end{aligned} \quad (35)$$

Power spectrum. Recall that the discrete-time filter transfer function is $H(z^{-1})$, so that the power spectra $S(\cdot)$ of the input and output signals are related by

$$S_y(e^{j\omega T}) = |H(e^{j\omega T})|^2 S_x(e^{j\omega T}) + \sigma^2 \quad (36)$$

where the squared amplitude response of the filter is easily shown to be

$$|H(e^{j\omega T})|^2 = \frac{(1 - \beta)^2}{1 - 2\beta \cos(\omega T) + \beta^2} \quad (37)$$

Once the estimated power spectrum $S_y(\cdot)$ has been fitted, the noise variance can be subtracted to yield a power spectrum which, when divided point-by-point by $|H(\cdot)|^2$, will yield the Markov signal's spectrum.

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The preferred storage medium is a 5.25 or 3.5 inch disk in MS-DOS format, although other systems are welcome, e.g., Macintosh (in this case, save your file in the usual manner, do not use the option 'save in MS-DOS format'). *After final acceptance, your disk plus one final, printed and exactly matching version (as a printout) should be submitted together to the accepting editor. It is important that the file on disk and the printout are identical.* Please specify the type of computer and word-processing package used (do **not** convert your textfile to plain ASCII). Ensure that the letter 'l' and digit '1' (also letter 'O' and digit '0') have been used properly, and format your article (tabs, indents, etc.) consistently. Characters not available on your word processor (Greek letters, mathematical symbols, etc.) should not be left open but indicated by a unique code (e.g. *gralpa*, @, #, etc., for the Greek letter α). Such codes should be used consistently throughout the entire text. Please make a list of such codes and provide a key. Do not allow your word processor to introduce word splits and do not use a 'justified' layout. Please adhere strictly to the general instructions on style/arrangement and, in particular, the reference style of the journal. Tables and illustrations will be handled conventionally; the latter should be submitted as original drawings or glossy prints. Further information may be obtained from the Publisher.

References

References in the text should start with the name of the author(s), followed by the publication date in brackets, e.g. Jones (1970) has shown the importance of... or... has been described (Jones, 1970; Brown and Jones, 1971; Chin et al., 1972)...; using date order. All references cited in the text should be listed at the end of the paper on a separate page (also double-spaced) arranged in alphabetical order of first author. All items in the list of references should be cited in the text and, conversely, all references cited in the text must be presented in the list. Literature references must be complete, including initials of the authors cited, year, title of paper referred to, abbreviated title, volume, and first and last page numbers of the article, in a periodical. The abbreviations of journal titles should conform to those adopted by *List of Serial Title Word Abbreviations*, CIEPS/ISDS, Paris, 1985, ISBN 2-904-938-02-8. (See example 2.) The form of literature references to books should be: author, initials, year, title of book, publisher and city, and page number referred to. (See example 1.) References to authors contributing to multi-author books or to proceedings printed in book-form should be similar to those for books. (See example 3.)

Examples:

Bureš, J., Burešová, O. and Huston, J.P. (1983) *Techniques and Basic Experiments for the Study of Brain and Behavior*, 2nd edn., Elsevier, Amsterdam, 326 pp.

Langston, J.W., Irwin, I., Langston, E.B. and Forno, L.S. (1984) The importance of the '4-5' double bond for neurotoxicity in primates of the pyridine derivative MPTP, *Neurosci. Lett.*, 50: 289-294.

Sofroniew, M.V. (1983) Morphology of vasopressin and oxytocin neurones and their central and vascular projections. In B.Z. Cross and G. Leng (Eds.), *The Neurohypophysis: Structure, Function and Control*, Progress in Brain Research, Vol. 50, Elsevier, Amsterdam, pp. 101-114.

Tables and figures

Tables should be compiled on separate sheets and numbered in Roman numerals. Figures included in the text should be prepared in black ink on suitable board or on graph paper with blue or faint grey rulings; graduation will not be reproduced. Drawings should be completely lettered, the size of the lettering being appropriate to that of the drawing, taking into account the necessary reduction in size (preferably not more than one third). The page format should be considered in designing the figures. The figures should be numbered in Arabic numerals. Photographs must be of good quality, printed on glossy paper.

Colour reproduction

Reproduction in colours will have to be approved by the Editors. The extra costs of colour reproduction will be charged to the author(s). Colour figures should be submitted as separate prints and not be mounted on cardboard. Professional labelling is preferred, but if this is not possible, an overlay displaying the desired labelling, should be provided with the figures. Please note that so-called press-on lettering should be avoided since this is likely to get damaged during handling and photographic processing of the prints. Slides taken from labelled prints are also acceptable.

Tables, figures and photographs should be clearly marked on the reverse side with the number, author's name and orientation (top); use a soft pencil or preferably a felt-tipped pen for marking photographs. Legends should be supplied on separate sheets.

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No changes in, or additions to, the manuscript will be allowed at this stage.

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