

Chapter 8

Analysis of whole cell currents to estimate the kinetics and amplitude of underlying unitary events: relaxation and ‘noise’ analysis

PETER T. A. GRAY

1. Introduction

The membrane currents evoked in voltage-clamped cells by a voltage step or by a pulse of neurotransmitter, released from nerve terminals or applied *in vitro*, are made up of the sum of many small unitary currents that flow through ion channels, aqueous pores in the cell membrane. Such summed, macroscopic, currents contain a component of ‘noise’ that results from the addition of many independent, randomly occurring unitary events. The time course of the evoked currents reflects the kinetics of the underlying channels and the channel kinetics determine the rate at which the relaxation to a new equilibrium occurs after a perturbation, such as a step of membrane potential.

The first direct evidence that these whole cell currents resulted from the summation of many smaller unit currents, flowing through ion channels, came from studies of the membrane voltage noise evoked by acetylcholine (ACh) at the frog neuromuscular junction, by Katz and Miledi (1970). Since that time more refined analysis of the noise components of current signals has allowed the amplitude and mean lifetime of the ACh activated channels at the frog neuromuscular junction (NMJ) to be measured (Anderson & Stevens, 1973); in turn allowing estimation of an upper limit of the lifetime of the pulse of ACh in the synaptic cleft (Magelby & Stevens, 1972). These techniques and relaxation analysis following a voltage step have also allowed investigation of the mechanisms of blockade of ion channels (Adams, 1977; Colquhoun *et al.* 1979).

More recently patch clamp techniques have allowed the kinetic analysis of both voltage-gated and transmitter activated conductances to be carried much further, as is described elsewhere in this book (Chapters 4 to 7). Though patch clamp studies can in principle provide much greater detail about the mechanisms of a conductance change voltage clamp techniques remain a valuable tool under many circumstances. Firstly, by their nature, studies of macroscopic currents involve the investigation of the averaged behaviour of all active ion channels in the cell membrane. By comparing the results of noise analysis with predictions obtained from single channel analysis it is

possible to check that the observed single channel behaviour can indeed explain the behaviour of the whole cell. This may not happen if, for example, more than one population of channels is present but unevenly distributed so that a single type is preferentially present in patches used for analysis, or as a second example, if a channel population has too small an amplitude to be resolved in single channel recordings but contributes appreciable current. Estimates of single channel amplitude from noise analysis that are much smaller than those obtained from single channel recording could be indicative of such problems, though they could also arise, for example, if a high frequency component of channel 'flicker' were resolved in single channel records, but was too fast to be resolved by whole cell noise recording. In addition, there are situations in which analysis of macroscopic currents may reveal details that single channel analysis cannot, for example, when the single channel amplitude is too small to be resolved (e.g. Gray & Attwell, 1985).

In general these forms of analysis of macroscopic signals provide information about two aspects of the underlying unitary events, their amplitude and kinetics. Two forms of analysis will be considered here in detail, (1) 'Noise', or fluctuation, analysis allows determination of information about both amplitude and kinetics from analysis of the fluctuations of a signal around the mean level. All electrical systems give rise to noise, for example 'shot' noise due to flow of charges in electronic components. The analysis of noisy signals was originally developed in relation to noise sources in electrical circuits and applied to biological systems by Katz & Miledi (1970). One of the principal difficulties to be faced when performing 'noise analysis' is the isolation of the biological noise from unwanted noise signals, such as shot noise in the recording circuitry and periodic interference such as 50 Hz mains and radio. (2) Whereas noise analysis gives information about the amplitude and time course of the underlying unitary events relaxation analysis of the kinetics of re-equilibration after a voltage or concentration step produces information solely about the kinetics of the events. In either case the data is analysed by comparing the observed data with the predictions of models of the unitary event, e.g. unitary current amplitude and channel kinetics.

While the signals analysed most frequently are membrane currents that result from the combination of single channel currents flowing through a population of ion channels, noise analysis can be applied to other forms of signal. For example the initial electrophysiological application of noise analysis by Katz & Miledi (1970) was a determination of the pulse of membrane potential that resulted from the opening of a single ACh activated channel at the frog NMJ. In general, however, meaningful kinetic information is much harder to obtain from voltage recordings, as the kinetics of the signals are determined by the effects of cell capacitance if the cell is not voltage clamped.

2. Using noise analysis to estimate unitary amplitudes

Stationary noise analysis

Noise analysis is based upon the fact that a steady state signal that is made up from a population of randomly occurring identical unitary events, such as single channel

currents, exhibits fluctuations, or noise, about its mean level. Analysis of these fluctuations allows the properties of the underlying events to be investigated. Fig. 1 illustrates how the combination of a large number of unitary current events produces a signal that has both a DC component and fluctuates about that level.

Determination of the amplitude of the unitary events from the noise signal requires calculation of the mean and variance of the signal. The variance of the signal is given by:

$$\text{var}(x) = \frac{1}{N} \sum_{i=1}^N (x_i - \bar{x})^2 \tag{1}$$

and the mean (\bar{x}) by:

$$\text{mean}(\bar{x}) = \frac{1}{N} \sum_{i=1}^N x_i \tag{2}$$

where N is the number of points sampled.

In practice the variance of a recorded signal consists of both the biological noise, the required signal, and background noise, for example instrument noise. However, the variance of two summed signals is given by:

$$\text{var}(a+b) = \text{var} a + \text{var} b + \text{covariance}(a,b).$$

Therefore, provided that the biological noise is independent from other noise sources (so that covariance (a,b) = 0) then the test variance is obtained simply by subtracting

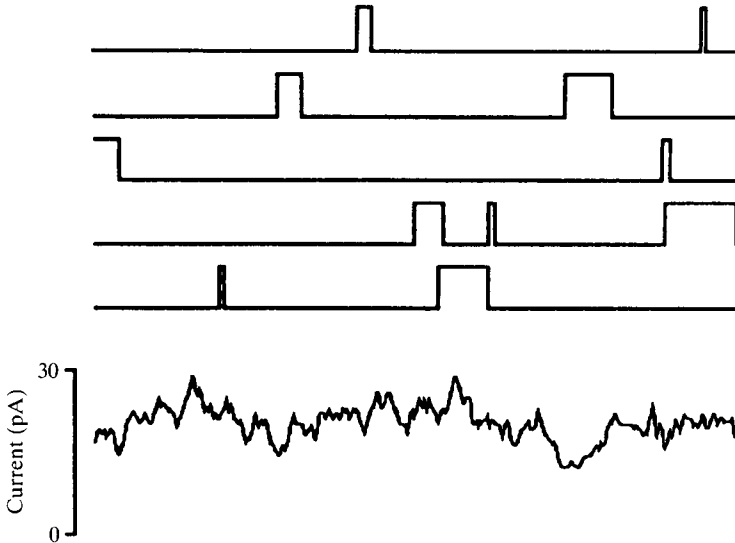


Fig. 1. An illustration of the way in which a large number of single channels opening randomly produce a noisy signal in addition to a DC current component. The upper five traces show computer generated current traces, modelled assuming a single channel current of 1 pA and a two-state model with $\alpha=200 \text{ s}^{-1}$ and $\beta=50 \text{ s}^{-1}$. The bottom trace is the sum of 100 such stretches of modelled single channel activity.

the background variance, which may be obtained from a control recording, from the total variance.

The relationship between the variance and mean and the amplitude of the unitary events depends upon the nature of those events. When studying the voltage noise generated by ion channels at the frog NMJ Katz and Miledi (1970, 1972) applied Campbell's theorem, which describes the noise generated by the summation of 'shot' events, each described by a function $f(t)$. In this case the mean and variance are given by:

$$\text{mean} = n \int_{-\infty}^{+\infty} f(t) dt \quad (3)$$

$$\text{var} = n \int_{-\infty}^{+\infty} f^2(t) dt \quad (4)$$

where n = frequency of events.

In the particular case of membrane voltage noise, if the unitary events are assumed to have the same amplitude then $f(t) = ae^{-t/\tau}$; where a is the amplitude and τ is the membrane time constant (this describes an elementary voltage 'blip' similar in waveform to the m.e.p.p., but of smaller amplitude). In this case the mean and variance are given by:

$$\text{mean}(V) = na\tau \quad (5)$$

$$\text{var}(V) = \frac{na^2\tau}{2} = \frac{a \cdot \text{mean}(V)}{2} \quad (6)$$

and the unitary amplitude is given by:

$$a = 2 \frac{\text{var}(V)}{\text{mean}(V)} \quad (7)$$

Alternative assumptions discussed by Katz and Miledi (1972) are when the unitary events had random amplitude or that they had different waveforms, $f(t)$.

Where the signal is current noise from a cell at constant voltage, i.e. data from voltage clamp recordings, then, provided that a single type of channel contributes to the noise, the mean current is given by:

$$\text{mean}(I) = NP_0i \quad (8)$$

where N is the number of channels, i the unit conductance and P_0 the steady state open probability (which will be a function of, for example, neurotransmitter concentration and membrane potential). If the channel may be only in an open or a closed state then, from binomial theory, the current variance is:

$$\text{var}(I) = Ni^2P_0(1-P_0) \quad (9)$$

where $(1-P_0)$ is the probability of the channel being closed. Thus:

$$\frac{\text{var}(I)}{\text{mean}(I)} = i(1 - P_o). \quad (10)$$

The plot of variance against mean gives a parabolic relationship as P_o varies from 0 to 1; however, for low values of P_o (i.e. $P_o \ll 1$) the relationship simplifies to:

$$i = \frac{\text{var}(I)}{\text{mean}(I)}. \quad (11)$$

Data preparation for noise analysis. The unit amplitude of an underlying unitary signal is most easily estimated by obtaining the mean and variance from a stretch of signal recorded under steady state conditions, i.e. in which the mean amplitude is constant, or at least changing slowly, over the duration of the recording. Once such a record has been obtained it can be analysed using a hardwired variance meter, in which case the mean and variance can be measured by hand from a chart record of the signal and its variance, and then plotted. Alternatively the signal may be sampled by a computer and analysed using software to calculate the variance, display the plot and fit a slope or parabola as needed (see Chapter 9). In practice the latter method is to be preferred, and with laboratory computer equipment becoming ever cheaper there are no longer any advantages to using a variance meter. In either case the raw data should be recorded on a DC tape recorder such as an FM or DAT recorder to allow gain and filter settings to be selected after completion of the experiment.

On play-back for analysis two signals must be prepared from the original recording (Fig. 2A). Firstly, a low pass filtered, low gain signal is needed for the measurement of mean current. Low pass filtering is essential to remove high frequency noise components that may obscure the biological noise. Noise generated by channel activity drops off at high frequencies, while, in contrast, electrical noise sources in the recording set-up increase with frequency, so substantial benefits in signal to noise ratio are obtainable by careful low pass filtering. Secondly, a high gain signal that has been both low and high pass filtered is needed for the calculation of variance. The high pass filtering of the second trace AC couples it, so that the signal contains only deviations from the mean level. This allows the signal from which the variance will be calculated to be amplified to a high gain, ensuring full use of the input resolution of the laboratory interface (see Chapter 9). The amplified signal should be monitored on an oscilloscope as it is sampled to ensure that it is sufficiently amplified to be well resolved, but not so large that it overshoots the input range of the ADC.

The choice of filter and sample frequencies must be made to ensure that the filter pass band used for the AC coupled trace encompasses all significant frequencies in the signal. This will depend upon the kinetics of the channels. If, for example, the low pass filter is set too low, then a significant amount of high frequency signal noise may be lost; this will result in an underestimate of the variance, and hence of i . The low pass filter setting must also be selected to ensure that 'aliasing' of the data does not occur. A set of data points sampled at frequency f points/sec cannot unambiguously represent a signal that contains periodic frequencies greater than $f/2$. It is impossible

to distinguish, in the sampled record, between a signal with a frequency below $f/2$ and a signal with a frequency the same amount above $f/2$. This is known as signal aliasing, and the frequency $f/2$ is known as the Nyquist frequency (see Chapter 16). It is essential that the signal is filtered at $\frac{1}{2}$ the sample rate, or below, in order to prevent distortion of both estimates of i and of any kinetic information that is obtained. For this reason Butterworth filters which have a very sharp roll off, but which may distort transient signals (see Chapter 16), are generally used for noise analysis.

In general, where the kinetics of the underlying events are not already well

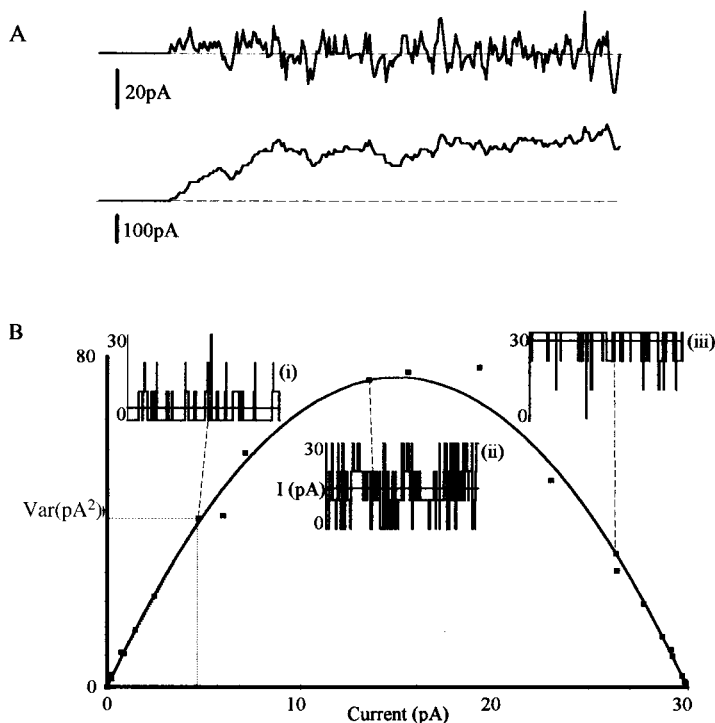


Fig. 2. Analysis of stationary current fluctuations. (A) Preparation of the data. The bottom trace shows data generated by a model that mimics the application of agonist to a cell possessing 60 channels, each of which have one closed state and one open (agonist bound) state in which an outward current of 10 pA passes under the experimental conditions. As the agonist washes on channels open and a noisy signal, fluctuating about a steady state, is evoked. The upper trace shows the same data AC coupled by high pass filtering at a higher gain. In both traces the zero current level is shown by the horizontal dashed line. (B) Current variance plotted against mean current for data generated by a model in which 3 identical channels are recorded from under a range of conditions. The channels have two states, a closed state and an open state, which carries a current of 10 pA under the experimental conditions. Data were generated for steady state open probability values ranging from 0 to 1, by varying the values of the opening rate (α) and the closing rate (β), used to generate the current records. The continuous line gives the predicted relationship between variance and mean current, calculated by the equation:

$$i\bar{i} - \bar{i}^2/N,$$

where $i = 10$ pA and $N = 3$.

The three inset traces show samples of the generated data, linked to their corresponding points on the variance/mean current plot by dashed lines. The mean current level is shown in each case by a continuous horizontal line.

described, it is better to perform both a kinetic and an amplitude analysis of the data, even if only the amplitude is of interest. This is so for the reason given above, i.e. that sample rates and filter frequencies must be chosen so that all significant frequency components in the signal are included in the variance calculation. This is hard to ensure unless the frequency characteristics of the signal are analysed. If a kinetic analysis is also carried out then an additional check on the estimated single channel current amplitude may be made from the parameters of the Lorentzian function fitted to the power spectrum (see below).

An additional advantage obtained by using a computer for the analysis is that the data may be readily edited. In general the long stretches of steady state data that are needed for noise analysis will contain a number of artefacts, such as spikes caused by neighbouring equipment turning on or off, or lower frequency artefacts caused by vibration. If analysed unedited such artefacts will lead to an over-estimation of variance and hence i . Once data has been sampled by the computer it may be displayed and parts selected for omission from analysis.

One source of unwanted noise that is hard to eliminate is 50 Hz mains 'hum'. In general this frequency falls within the frequency range of interest, so cannot be filtered out. Low levels of mains noise that are not visible by inspection of the raw data may still be sufficient to generate a peak at 50 Hz (and sometimes at the harmonics 100 and 150 Hz) in a noise spectrum. For this reason particular care in eliminating mains pickup is required when recording data for noise analysis.

Data analysis. Once the variance has been calculated the unit current amplitude, i can be calculated. The initial slope of the relationship between variance and mean current described by equation 10 is i . Thus for currents that are small relative to the maximum, i.e. under conditions where the steady state open probability (P_o) of any channel is low, then the relationship between variance and mean current is a straight line with an intercept of 0. Under such circumstances i is given by equation 11, i.e.:

$$i = \frac{\text{var}(I)}{\text{mean}(I)}$$

and can be calculated directly from the data. Inset (i) in Fig. 2B illustrates such a case.

However, under conditions where the assumption that P_o is small does not hold then it is apparent that equation 11 will yield erroneous results. In such cases, or where it is not known if the assumption holds, it is necessary to obtain recordings at different mean currents and to produce a plot of variance against mean current to which a parabola can be fitted (Fig. 2B), or a straight line if it proves that the assumption of low P_o is accurate. Such data may be obtained by applying different agonist concentrations, as is modelled in Fig. 2B, or by analysing the rising or falling phases of a response (e.g. Gray & Attwell, 1985). In the latter case it is important that the rate of change in mean current should be sufficiently slow that the change in mean current during the period over which each variance sample is calculated is small.

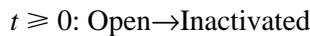
Non-stationary noise analysis

The analysis described above requires recordings to be made of noise signals whose characteristics remain steady over a period of time, or at least where the changes are slow relative to the length of the periods over which variance and mean current are calculated (for example analysis of transmitter activated noise where the transmitter is applied at low concentration to the preparation by bath perfusion). However, some events have properties that change too rapidly for such methods to be applied. Most notably, many voltage activated conductances activate and inactivate rapidly after a voltage step. This makes it impossible to use stationary noise analysis to estimate the single channel current amplitude. An alternative approach is possible and was described by Sigworth (1980). The method relies on recording a large number of responses to an identical stimulus, in this case a voltage step, though the method has also been applied to synaptic currents by Traynelis *et al.* (1993). The variance is calculated at identical time points after the step by summing many consecutive responses. Thus, the mean and variance are:

$$\bar{I}(t) = \frac{1}{n} \sum_{k=1}^n y_k(t) \quad (12)$$

$$\sigma^2(t) = \frac{1}{n-1} \sum_{k=1}^n (y_k(t) - \bar{I}(t))^2 \quad (13)$$

where $\bar{I}(t)$ is the mean current at time t for all n records, $\sigma(t)$ the variance at time t and $y_k(t)$ is the signal amplitude of the k^{th} record at time t after the stimulus or start of the step. This process is illustrated in Figs 3 and 4. Fig. 3A shows three traces generated by computer simulation to illustrate the inactivation phase of a voltage activated conductance which has an outward single channel current of 10 pA. For simplicity it is assumed that the activation by the voltage step at time 0 is instantaneous, the open probability $P_o=0.5$ immediately after the step and that open channels only close to an inactivated state from which they do not reopen. The model for each channel can be represented as:



with a rate constant of 40 s^{-1} . The records are from a patch of membrane containing 3 such channels, though in practice such an analysis would generally be performed on preparations containing many more channels. Fig. 3B shows the mean current obtained by averaging 250 traces, and Fig. 3C illustrates the calculation of the deviation, i.e.

$$y_k(t) - \bar{I}_k(t)$$

by subtracting the mean current averaged from all traces from the k^{th} raw data trace. In practice, to allow for slow changes with time during the course of a long experiment, such records would be averaged in groups so that mean current and variance were calculated repeatedly for several sets of data. This would reduce the

likelihood of over-estimation of the variance due to slow drift in experimental parameters.

Once the records have been averaged, and the deviations calculated then the variance for each time point can be calculated. Fig. 4A shows the variance plotted against time for the 250 simulated traces. By plotting variance for each time point against the mean current for the same time point (Fig. 3B) a plot of variance against mean current is obtained (Fig. 4B). In the case modelled, the initial value of P_o was 0.5 so i cannot be estimated from the initial slope of the parabola, but must be obtained by fitting equation 10 to the data. In practice, when fitting such data it is often convenient to rearrange equation 10 by substituting

$$P_o = \frac{\bar{I}}{N \cdot i}$$

giving:

$$\text{var } I = i\bar{I} - (\bar{I}^2/N)$$

(where N is the number of channels) to obtain estimates of parameters i and N . The

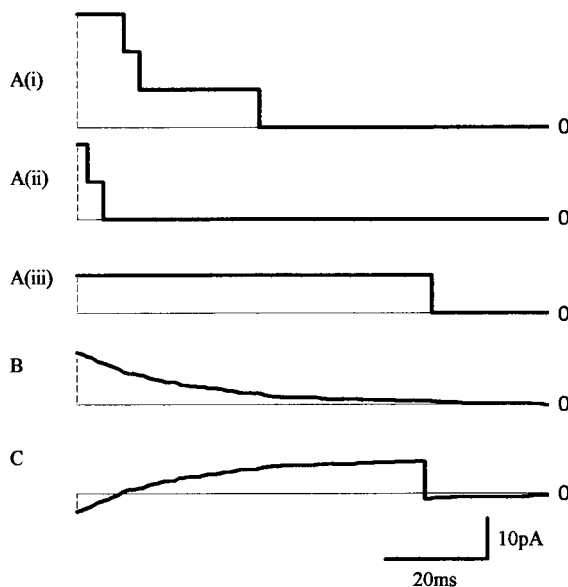
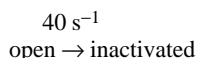


Fig. 3. Non-stationary noise analysis - stage 1. Data were generated from the following model assuming that after a voltage step channels have a P_o of 0.5 and that only one transition can occur:



i.e. the channels can only close to an inactivated state, from which they do not reopen. The channel lifetimes are randomly distributed with a mean of 25 ms. 250 sets of data were generated from this model, assuming a patch containing 3 such channels, and that the experimental conditions gave an outward current of 10 pA through the open channel. Three of these traces are shown in A(i)-A(iii). B shows the mean current in response to the step, obtained by averaging all 250 sweeps. Trace C shows a sample trace in which the mean current has been subtracted from one of the sweeps, the data shown is that from trace A(iii).

open probability at each current level can be obtained by substitution ($I=NiP_o$). In Fig. 4B this parabola has been superimposed on the data, with $i=10\times 10^{-12}$ A and $N=3$.

For the same reasons as are described above in relation to steady state analysis it is important that the raw data can be edited prior to analysis to eliminate artefacts, and that allowance is made for variance that originates from sources other than those of interest. If the channels being studied can be blocked fully by an applied drug then a control can be obtained by repeating the experiment in the presence of that drug, for example when studying sodium channels they may be blocked with TTX.

3. Kinetic analysis of macroscopic currents

Relaxation analysis

When a population of channels is perturbed by an event such as a pulse of agonist or a step change in membrane potential, the current through those channels will change (relax) to a new equilibrium level at a rate which reflects the underlying kinetics of the channels. The relationship between the rate constants for the transitions of the channels between states and the current through a large number of channels can be derived from the law of mass action. In the simple case of a voltage gated channel

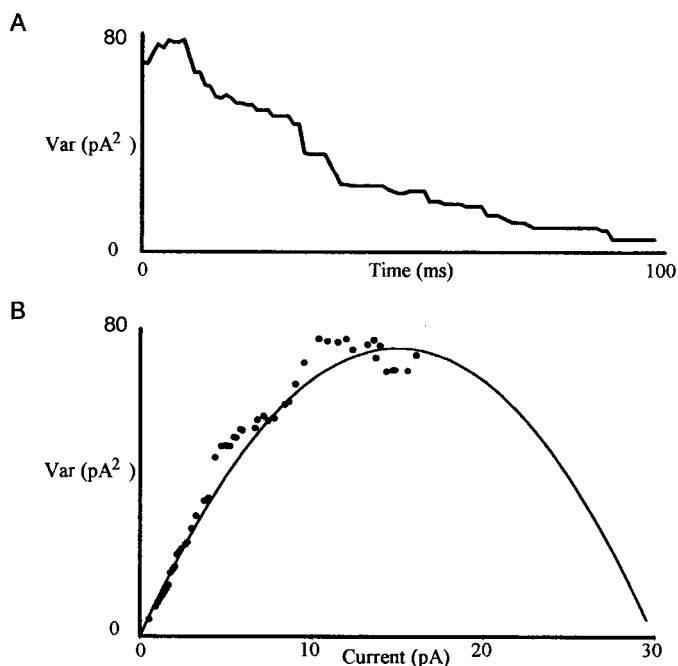


Fig. 4. Non-stationary noise analysis - stage 2. After subtraction of the mean current from the set of traces the variance at each time point is calculated, allowing a plot of variance against time to be made (A). Finally, the values for variance are replotted against mean current (B). A parabolic curve can be fitted to this data. In this case the values used for the parameters of the curve are unit current (i) = 10 pA, number of channels (N)=3.

having only two states, closed (C) and open (O), the rate constants for the opening (β) and closing (α) being voltage dependent, the analysis is as follows:



Following a step change in potential, which modifies the rate constants, the change in probability of the channel being in the open state (P_o) with time after the step is given by the sum of the fluxes into and out of the open state, i.e.:

$$\frac{dP_o}{dt} = \beta P_c - \alpha P_o \quad (14)$$

where α and β are the rate constants at the new potential. Since the probability of being in the closed state $P_c=1-P_o$ this can be rearranged as the differential equation:

$$\frac{dP_o}{dt} = \beta - (\alpha + \beta)P_o \quad (15)$$

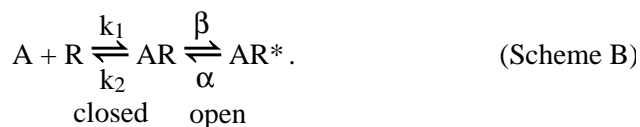
which integrates to give:

$$P_o(t) = P_o(\infty) + [P_o(0) - P_o(\infty)] e^{-t/\tau} \quad (16)$$

where $\tau = 1/(\alpha + \beta)$ and $P_o(\infty) = \beta/(\alpha + \beta)$, the steady state probability of being in the open state.

Thus relaxation to a new steady state after a perturbation follows an exponential time course having a time constant of $1/(\alpha + \beta)$. Where P_o is low (i.e. $\alpha \gg \beta$) the rate constant of the relaxation gives an estimate of the mean channel lifetime ($1/\alpha$).

In the case of transmitter activated channels the simplest possible scheme is slightly more complex, as both binding and opening must be modelled, i.e.:



However, if binding is assumed to be much more rapid than opening, so reequilibration is determined by the opening reaction alone, then this can be simplified to:



where $\beta' = \beta.A/(A + K)$, with $K = k_2/k_1$ and $A =$ concentration of agonist. It can be seen that with this assumption $\beta' = \beta$ times the steady state probability of the $A+R \rightleftharpoons R$ equilibrium being in the AR state, so the opening rate depends on the transmitter concentration. In this

case equation 16 still holds, with β' replacing β . Similarly, for a transmitter activated channel, where the transmitter concentration is low ($\alpha \gg \beta'$) the relaxation will still have a time constant that approximates to the mean channel lifetime ($1/\alpha$).

This result is made use of in voltage-jump relaxation experiments and has been applied to a number of preparations in which the channel opening and closing rate constants are voltage dependent. It has been particularly useful in cases where the opening and closing rate constants of a transmitter activated channel are voltage dependent (such as the nicotinic ACh channel of the endplate, e.g. Adams, 1975; Neher & Sakmann, 1975).

Though concentration jumps would in principle perturb the equilibrium proportion of open transmitter activated channels, allowing kinetic information to be obtained in a similar way, voltage-jump relaxation experiments have in general proved more useful as voltage clamp circuits allow good control of membrane potential. In contrast, rapid concentration jumps are difficult to produce on even a single cell because of the time

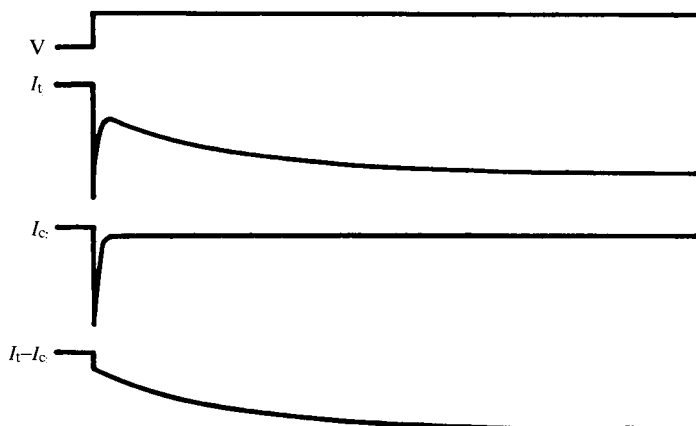


Fig. 5. Illustrates the protocol of a voltage-jump experiment. The upper trace (V) represents the voltage step and I_t , (the total current) a hypothetical recording of the current evoked in response from a preparation such as the frog neuromuscular junction in presence of ACh. Four components to the response can be seen. The first two of these components are indistinguishable in this trace. They are both step changes in the current level, due to the increased driving force of the voltage step causing a larger current to flow both through those channels that were open at the instant of the voltage step and through the leakage conductance. Thirdly, there is a fast transient current superimposed on the step change, this is the current that flows into the cell capacitance as a result of the change in potential gradient. Lastly, there is a slow exponential relaxation to a new steady current level. This is due to the proportion of open channels changing to a new level with a time constant of $1/(\alpha + \beta')$, where the values of α and β' are those corresponding to the new potential. The effects of the leakage conductance and cell capacitance are often corrected for as shown in the lower two traces. In the case of transmitter activated channels the jump protocol can be repeated in the absence of agonist. In this case the recorded current (I_c) is in principle the sum of the same leakage and capacitive components that are present when the agonist is. By subtracting the currents in the presence and absence of agonist the current that is carried by the transmitter activated channels in response to the voltage step is found ($I_t - I_c$). Similar corrections are possible when studying voltage activated channels by comparing the response to voltage steps in opposite directions. If the channels studied are activated by voltage steps of one polarity but not the other then again the leakage and capacitive components can be subtracted by adding currents evoked by jumps in both directions.

taken to diffuse across 'unstirred' regions adjacent to the surface (10-20 ms on single neurones). Furthermore, the binding of the drug to the receptors being studied may further slow diffusion in thick preparations (see Colquhoun & Ritchie, 1972). Rapid concentration changes have proved possible using outside out patches, where the unstirred layer is small, and diffusion times can be reduced to about 1 ms. In combination with fast perfusion systems this approach has allowed study of the response of transmitter activated channels to step changes in concentration (Franke *et al.* 1987; Lui & Dilger, 1991; Maconochie & Knight, 1989). A special case in which a concentration jump can be said to occur is in the initiation of the endplate current, when rapid release and hydrolysis of the pulse of ACh means that it is short lived relative to the channel activated lifetime and so channels do not in general reopen once closed (Magelby & Stevens, 1972). Thus the timecourse of the e.p.c. is determined by the duration of individual activations of the receptor (see Chapter 7).

Fig. 5 shows the protocol of a voltage-jump experiment on nicotinic ACh receptors. At the holding potential (V_H) a steady current flows in the presence of a low and constant agonist concentration. As shown in the upper trace, the voltage is stepped to a test potential (V_t) after a delay. This evokes a step change in the recorded current (I_t), with a superimposed rapid capacity transient. Following these changes there is a slow increase in the inward current to a new steady state level. The steady state change consists of two components, the change in current flowing through the leak conductance and the change in current flowing through that proportion of channels open at equilibrium at V_H . The slow change in the current signal represents the relaxation of the population of channels towards a new equilibrium open probability, different from that at V_H as both the opening and closing rate constants are voltage dependent. The time constant of the relaxation is $1/(\alpha + \beta')$, at V_t . In practice the capacity transient and channel related relaxation are often not so clearly separated so it is generally necessary to record the response to the voltage step in both the presence and absence of the agonist. The difference of the two signals then, in principle, represents only the current flowing through drug activated channels (lower traces). In practice many such responses will be averaged to minimise the effects of noise sources.

Where data cannot be fitted by a simple model that has only two resolvable rate constants, e.g. if there is inactivation or desensitisation, the relaxation may need to be fitted with the sum of two or more exponentials.

Noise analysis

Theoretical basis. Noise analysis can be used to investigate the kinetic properties of unitary events. It is intuitively clear that the time or frequency characteristics of the noise must reflect, in some way, those of the underlying events. This relationship is most easily understood by considering the autocovariance of the signal. The autocovariance measures the correlation between values of a noisy signal measured at time intervals of Δt apart, and for a signal with a mean value of zero it is given by

$$C(\Delta t) = \Sigma \frac{x(t).x(t + \Delta t)}{n - 1} \quad (17)$$

where $x(t)$ is the signal value at any time t , $x(t + \Delta t)$ is the value a given time increment (Δt) later and n is the number of values of x sampled. For a current signal the autocovariance would be measured as shown in Fig. 6. The current amplitudes are measured relative to the mean current level. Thus if Δt is very large relative to the cycle length of the major frequency components of the noise signal then the pairs of current values will be uncorrelated and the value of $C(\Delta t)$ will on average be very small. In contrast, if Δt is very small the pairs of current values will be well correlated, i.e. similar in value, as little change in the current signal would be expected if the time increment is short (compared to the cycle lengths of the predominate frequency components in the signal), and the value of $C(\Delta t)$ will be high. Thus $C(\Delta t)$ will be high for small values of Δt and approach zero as Δt approaches infinity. Clearly the way in which $C(\Delta t)$ falls with increasing time increments (Δt) will be determined by the frequency characteristics of the noise.

What is the relationship between $C(\Delta t)$ and the single channel kinetics? Firstly, we need only consider the behaviour of a single channel, if the N channels present have the same properties and behave independently of each other the autocovariance for all N channels is simply $N \times$ the autocovariance of a single channel. Thus

$$C(\Delta t) = N \Sigma \frac{x(t).x(t + \Delta t)}{n - 1} \tag{18}$$

$$= N \times \text{mean of } [i(t) . i(t + \Delta t)]$$

where $x(t)=i(t)$ the current through a single channel at time t and $i(t+\Delta t)$ is the current in the same channel at $t+\Delta t$. As the single channel current is either i or zero, $i(t).i(t + \Delta t)$ has a value of either i^2 or zero. The value is i^2 if the channel is open at both t and at

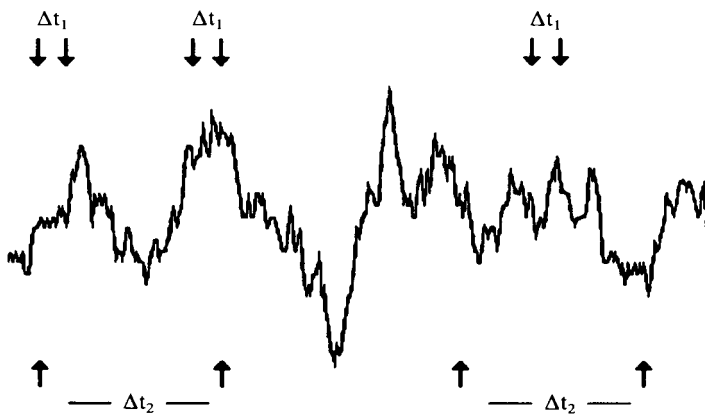


Fig. 6. The measurement of autocovariance. The autocovariance is calculated by taking the product of the current value at time t and the value at time $t + \Delta t$. The sum of these products for each value of t is then found. It is apparent that when the time interval is small relative to the major frequency components of the noise (Δt_1) then the autocovariance will be high (the values within each pair will be highly correlated). However, if the interval is long (Δt_2) then the autocorrelation will be low.

$t + \Delta t$. For a single channel the mean value of $i(t).i(t + \Delta t)$ is given by the probability of the channel being open at *both* times t and $t + \Delta t$ multiplied by i^2 . Thus

$$C(\Delta t) = Ni^2P[\text{open at } t \text{ and open at } t + \Delta t]. \quad (19)$$

$P[\text{open at } t \text{ and open at } t + \Delta t]$ can be restated by the rule that the probability of both of two events is the product of the probability of each of them. $P[\text{open at } t \text{ and open at } t + \Delta t]$ is the product of $P[\text{open at } t]$ and $P[\text{open at } t + \Delta t | \text{open at } t]$. The second term is the conditional probability that the channel is open at $t + \Delta t$ given that it was open at t . Thus:

$$\begin{aligned} C(\Delta t) &= Ni^2P[\text{open at } t].P[\text{open at } t + \Delta t | \text{open at } t] \\ &= Ni^2P_o. P[\text{open at } t + \Delta t | \text{open at } t] \end{aligned} \quad (20)$$

as $P[\text{open at } t]$ is simply the open probability, P_o . $P[\text{open at } t + \Delta t | \text{open at } t]$ is the same as the probability of the lifetime being $\geq \Delta t$ provided that there is a negligible probability of the channel closing and reopening during Δt . The statistical distribution of random lifetimes Δt with a mean value τ is:

$$P[\text{lifetime} \geq \Delta t] = e^{-\Delta t/\tau} \quad (21)$$

(see Chapter 7). Thus τ is the mean open lifetime ($=1/\alpha$ in Scheme C) and equation 20 can be rewritten as:

$$C(\Delta t) = Ni^2P_o e^{-\Delta t/\tau} \quad (22)$$

and since $\bar{I} = NiP_o$:

$$C(\Delta t) = \bar{I}i e^{-\Delta t/\tau}.$$

If the channel has an Ohmic I/V relation so $i = \gamma(V - V_{eq})$ where γ is the single channel conductance and $(V - V_{eq})$ the electrochemical driving potential then

$$C(\Delta t) = \bar{I}\gamma(V - V_{eq})e^{-\Delta t/\tau}. \quad (23)$$

Therefore the autocovariance of a current noise signal with gating described by Scheme C, when plotted against Δt , decays exponentially with a time constant of $1/\alpha$ (Fig. 7A).

Most commonly such data is presented not in the form of an autocovariance plot but as a power spectrum (Fig. 7B) in which the power carried by the signal at each frequency is plotted against frequency, f . The 'power' is effectively the (current)² flowing in a 1Ω resistor and the ordinate of the power spectrum has dimensions $A^2Hz^{-1} = A^2s$. This is the Fourier transform of the autocovariance plot, which gives us an expression in terms of frequency instead of time. The reason for doing this is that the handling of the data is simplified as the Fourier transform of the data can be easily obtained by the use of one of the fast Fourier transform computer routines available (see Chapter 9). The Fourier transform of $e^{-\Delta t/\tau}$ is

$$\frac{2}{1 + (2\pi f\tau)^2}$$

thus

$$G(f) = 4\bar{I}\gamma(V - V_{\text{eq}}) \frac{1}{1 + (2\pi f\tau)^2} \quad (24)$$

where $G(f)$ is the spectral density function; this has been multiplied by two to obtain the 'single sided' spectral density function (Bendat & Piersol, 1986). We can define $\tau = 1/2\pi f_c$, for convenience, thus

$$G(f) = 4\bar{I}\gamma(V - V_{\text{eq}}) \frac{1}{1 + (f/f_c)^2} \quad (25)$$

This is a Lorentzian function and when plotted on log/log coordinates as a power spectrum has a characteristic shape (Fig. 7B). The terminology applied to power spectra is important (see Chapter 16); f_c is the 'cut-off' or 'half-power' frequency, which is the frequency at which the power carried has dropped to $\frac{1}{2}$ of the zero frequency value [$G(0)$]. $G(0)$ is usually known as the 'zero frequency asymptote' as the zero frequency point is not measured (measurement of the zero frequency point would require infinite sample lengths). Beyond f_c the curve falls away with a slope of -2 which gives rise to the term ' $1/f^2$ noise' which is sometimes applied to signals that fit the high frequency slope of the Lorentzian function.

A simple Lorentzian spectrum is predicted by models of channel opening with single open and closed states. More complex models predict the sum of 2 or more Lorentzians, in general the number of Lorentzians = the total number of states (open and closed) -1 . However, one or more of these states may have very small amplitude and be unresolvable. For a full treatment of the theoretical basis of fitting data with the sum of multiple Lorentzians see Colquhoun and Hawkes (1977).

As discussed in the previous section, noise analysis can also provide information about the amplitude of the single channel currents that underlie the macroscopic current, the single channel current being obtained by dividing the variance of the signal by the mean current. The variance of a noise signal that follows a Lorentzian relationship is given by the area under the spectral density function. Thus the single channel conductance can be estimated from the integral of the fitted Lorentzian between zero and infinity, which can be shown to be

$$\text{var} = \frac{\pi G(0)f_c}{2} \quad (26)$$

where $G(0) = 4\bar{I}\gamma(V - V_{\text{eq}})$, the zero frequency asymptote. Thus

$$\gamma = \frac{\pi G(0)f_c}{2\bar{I}(V - V_{\text{eq}})} \quad (27)$$

as $\gamma = i/(V - V_{\text{eq}})$ from Ohm's law, where i is the unit current and $i = \text{var}/\bar{I}$ (equation 11), provided that P_o is low. If γ is to be estimated from the spectra it is essential that the

mean current is constant during the period over which all data used for calculation of spectra were collected.

In situations where the noise spectrum is fitted by the sum of more than one Lorentzian the conductance can be estimated from the sum of a series of terms relating to each of the Lorentzian terms, provided the assumption is made that all the

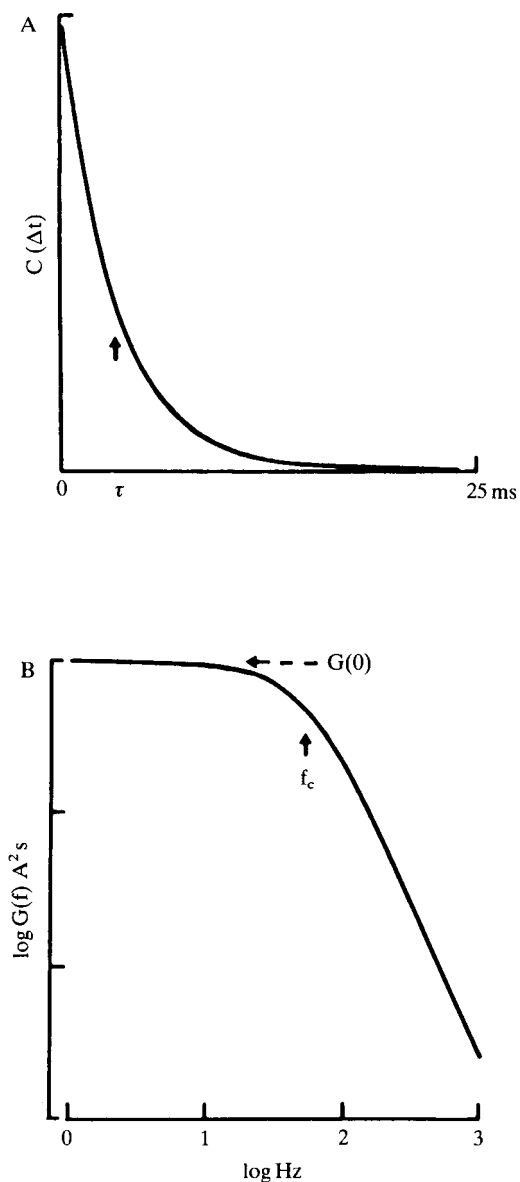


Fig. 7. The autocovariance of the noise signal produced by a 2 state model falls off exponentially as the time interval is increased (A). Generally the results of noise analysis are plotted in the form of a power spectrum on log/log coordinates (B). The form of the curve is a Lorentzian, the half-power frequency (f_c) = $1/2\pi\tau$, where τ is the time constant of the autocovariance plot (in this case 3.18 ms, so $f_c = 50 \text{ Hz}$).

open states (if more than one) have the same conductance, i.e. when the noise signal can be fitted by the sum of two Lorentzian components then γ can be estimated from

$$\gamma = \frac{\pi G(0)1f_{c1}}{2\bar{I}(V - V_{eq})} + \frac{\pi G(0)2f_{c2}}{2\bar{I}(V - V_{eq})}. \quad (28)$$

This approach to the estimation of γ may be useful at times, particularly where a significant fraction of the noise is outside the bandwidth of the recording equipment, provided there is good reason to be confident about the fitted model. An alternative approach to the problem of restricted bandwidth is to calculate the predicted proportion of unresolved variance, after fitting a spectrum, and to adjust the value of the directly measured current variance accordingly (Colquhoun *et al.* 1979).

For more complete treatments of the theoretical basis of noise analysis see Bendat & Piersol (1986), Colquhoun & Hawkes (1977), Conti & Wanke (1975), Neher & Stevens (1977) or DeFelice (1981).

Data preparation

Preparation of data for kinetic analysis using noise analysis is essentially the same as that described above when using noise analysis for investigating unit amplitudes. However, two additional factors must be considered. Firstly, the sampled noise signal will contain, in addition to the biological noise of interest, noise from a number of other sources. All electrical recording equipment generates noise, significant elements of which will depend upon the characteristics of the cell or other preparation being recorded from, in particular the cell capacitance will determine the amplitude of significant elements of noise. Furthermore, other channels or carriers in the cell membrane may generate signals, which themselves contain fluctuations. For these reasons, it is essential that allowance is made for those components of noise that are not generated by the events of interest. This is generally done by determining the spectrum of a control signal and subtracting this from the total spectrum prior to fitting any model. Where the events being studied can be readily turned on by application of some external factor, such as an agonist, then the net variance is readily calculated by subtracting the variance in the absence of the agonist from that in the presence of the agonist.

Secondly, the data must be split into records whose length depends upon the lower and upper frequencies required in the power spectrum. The lowest frequency point obtained is given by the inverse of the sample length, thus a 2 second long sample is needed if the minimum frequency point is to be at 0.5 Hz. This also determines the frequency resolution of the power spectrum, power values will be obtained for frequencies incrementing by the same value, every 0.5 Hz in the example. The high pass filter setting used to prepare the AC coupled record should be chosen to allow data at the lowest frequency point in the spectrum to pass unattenuated, but to attenuate all frequencies below this. The high frequency limit of the spectrum is the same as the Nyquist frequency, i.e. half the sample rate. In general, steady state stretches of both experimental and control data would be obtained. These would be

edited to remove artefacts, split into records and analysed. A number of spectra, usually 10–20, would be obtained for both sets of conditions and all spectra in each set averaged, to improve the signal to noise ratio of the final spectra. Finally the mean control spectrum is subtracted from the mean experimental spectrum before any curve fitting takes place.

Estimates of the parameters of a power spectrum are obtained by fitting Lorentzian curves to the data. In general this will be done using a least squares algorithm. Procedures for curve fitting and the estimation of errors are discussed in Chapter 9.

References

- ADAMS, P. R. (1975). An analysis of the dose-response curve at voltage clamped frog endplates. *Pflugers Arch.* **360**, 145-153.
- ADAMS, P. R. (1977). Voltage jump analysis of procaine action at frog endplate. *J. Physiol., Lond.* **268**, 291-318.
- ANDERSON, C. R. & STEVENS, C. F. (1973). Voltage clamp analysis of acetylcholine produced end-plate current fluctuations at frog neuromuscular junction. *J. Physiol., Lond.* **235**, 655-691.
- BENDAT, J. S. & PIERSOL, A. G. (1986). *Random Data: Analysis and Measurement Procedures*. New York: Wiley Interscience.
- COLQUHOUN, D., DREYER, F. & SHERIDAN, R. E. (1979). The actions of tubocurarine at the frog neuromuscular junction. *J. Physiol., Lond.* **293**, 247-284.
- COLQUHOUN, D. & HAWKES, A. G. (1977). Relaxation and fluctuations of membrane currents that flow through drug-operated channels. *Proc. R. Soc. Lond. B* **199**, 231-262.
- COLQUHOUN, D. & RITCHIE, J. M. (1972). The kinetics of the interaction between tetrodotoxin and mammalian nonmyelinated nerve fibres. *Molec. Pharmacol.* **8**, 285-292.
- CONTI, F. & WANKE, E. (1975). Channel noise in nerve membranes and lipid bilayers. *Q. Rev. Biophys.* **8**, 451-506.
- DEFELICE, L. J. (1981). *Introduction to Membrane Noise*. New York: Plenum Press.
- FRANKE, C., HATT, H. & DUDEL, J. (1987). Liquid filament switch for ultra-fast exchanges of solution at excised patches. *Neurosci. Lett.* **77**, 199-204.
- GRAY, P. T. A. & ATTWELL, D. (1985). Kinetics of light-sensitive channels in vertebrate photoreceptors. *Proc. R. Soc. Lond. B* **223**, 379-388.
- KATZ, B. & MILEDI, R. (1970). Membrane noise produced by acetylcholine. *Nature, Lond.* **226**, 962-963.
- KATZ, B. & MILEDI, R. (1972). The statistical nature of the acetylcholine potential and its molecular components. *J. Physiol., Lond.* **224**, 665-699.
- LUI, Y. & DILGER, J. P. (1991). The opening rate of ACh receptor channels. *Biophys. J.* **60**, 424-432.
- MACONOCHIE, D. & KNIGHT, D. E. (1989) A method for making solution changes in the submillisecond range at the tip of a patch pipette. *Pflugers Arch.* **484**, 589-596.
- MAGELBY, K. L. & STEVENS, C. F. (1972). A quantitative description of endplate currents. *J. Physiol., Lond.* **223**, 173-197.
- NEHER, E. & SAKMANN, B. (1975). Voltage-dependence of drug-induced conductance in frog neuromuscular junction. *Proc. Natl. Acad. Sci. USA* **72**, 2140-2144.
- NEHER, E. & STEVENS, C. F. (1977). Conductance fluctuations and ionic pores in membranes. *Ann. Rev. Biophys. Bioeng.* **6**, 345-381.
- SIGWORTH, F. J. (1980). The variance of sodium current fluctuations at the node of Ranvier. *J. Physiol. Lond.* **307**, 97-129.
- TRAYNELIS, S. F., SILVER, R. A. & CULL-CANDY, S. G. (1993). Estimated conductance of glutamate receptor channels activated during EPSCs at the cerebellar mossy fibre-granule cell synapse. *Neuron* **11**, 279-289.