Chapter 7

The interpretation of single channel recordings

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1. Introduction

The information in a single channel record is contained in the amplitudes of the openings, the durations of the open and shut periods, and in the order in which the openings and shuttings occur. The record contains information about rates as well as about equilibria, even when the measurements are made when the system as a whole is at equilibrium. This is possible because, as with noise analysis, when the recording is made from a small number of channels the system will rarely be *exactly* at equilibrium at any given moment. If, for example, 50 percent of channels are open at equilibrium this means only that over a long record an individual channel will be open for 50 percent of the time; an individual channel will never be '50 percent open'.

In this chapter we shall consider how various aspects of single channel behaviour can be predicted, on the basis of a postulated kinetic mechanism for the channel. This will allow the mechanism to be tested, by comparing the predicted behaviour with that observed in experiments. We shall also discuss the effect that the imperfect resolution of experimental records will have on such inferences.

2. Macroscopic measurements and single channels

Measurements from large numbers of molecules will be referred to here as *macroscopic measurements*. For example the decay of a miniature endplate current (MEPC) at the neuromuscular junction provides a familiar example of a measurement of the rate of a macroscopic process. Several thousand channels are involved, a large enough number to produce a smooth curve in which the contribution of individual channels is not very noticeable. In this case the time course of the current is usually a simple exponential. Other forms of macroscopic measurements of rate include (*a*) observations of the reequilibration of the current following a sudden change in membrane potential (*voltage jump relaxations*), and (*b*) reequilibration of the current following a sudden change in ligand concentration (*concentration jump relaxations*).

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In these cases too, it is common to observe that the time course of the current can be fitted by an exponential curve, or by the sum of several exponential curves with different time constants. This is exactly the result expected on the basis of the law of mass action if (*a*) the system exists in several discrete states (see Colquhoun & Ogden, 1986, for a discussion), and (*b*) the transitions between these states occur at a constant rate. Before going further the terms used to define rates will be described.

Rate constants and transition rates

The *transition rate* between two states always has the dimensions of a rate or frequency, viz s^{-1}. For a unimolecular reaction that involves only one reactant (e.g. a conformation change) the transition rate is simply the *reaction rate constant* defined by the law of mass action. The law of mass action states that the *rate* of a chemical reaction (with dimensions s^{-1}) is directly proportional to the product of the reactant concentrations at any given moment, the *rate constant* being the constant of proportionality.

For a binding reaction, which is bimolecular (both receptor and free ligand are reactants), the reaction rate constant has dimensions $M^{-1}s^{-1}$; the actual transition rate in this case is the product of the rate constant ($M^{-1}s^{-1}$) and the free ligand concentration (M) and will have dimensions s^{-1} as required. The assumption that the transition rates are constant, i.e. they do not change with time, involves the assumption that the free concentration does not change with time; this may not always be true.

When the assumptions are satisfied the number of exponential components will, in principle, be one less than the number of states (e.g. Colquhoun & Hawkes, 1977). We must now ask how these relatively familiar measurements of rates are related to what is observed with a single ion channel.

Consider the simplest possible example, a channel that can exist in only two states, open and shut. The states will be numbered 1 and 2 respectively, so the rate constant for transition from shut to open is denoted k_{21}, and that for transition from open to shut is denoted k_{12}. The mechanism is conventionally written as

(2.1)

This is simple for two reasons. Firstly it is simple because there are only two states, and secondly it is simple because these two states are distinguishable by looking at the experimental record. We can see from the record which state the system is in at any moment. In the simple mechanism in (2.1), the opening rate is k_{21} times the fraction of shut channels (shut channels are the only reactant that participates in the opening reaction). For a simple binding reaction, A+R $\rightleftharpoons$ AR (A=ligand molecule, R=receptor), the association rate constant, k_{+1} , has dimensions $M^{-1}s^{-1}$ so when it is multiplied by the free concentration of A (to which the association rate is

proportional) we get the actual transition rate for the forward reaction with dimensions s^{-1}.

Macroscopic behaviour

If the reaction in (2.1) is perturbed (e.g. by a sudden change in membrane potential or ligand concentration), the new equilibrium condition will be approached with a simple exponential time course (there is one exponential because there are two states). The rate of this process can be expressed as an *observed time constant*, τ, or as its reciprocal, the *observed rate constant* $\lambda=1/\tau$. These values are given by

$$\tau = 1/(k_{12} + k_{21})$$

or

$$\lambda = k_{12} + k_{21} \tag{2.2}$$

The equation that describes the time course contains an exponential term of the form $e^{-\lambda t}(=e^{-t/\tau})$. Fig. 1 shows an example, an endplate current for which τ=7.1 ms. The observed current at time t will be directly proportional to the fraction of channels that are open (state 1) at time t, and this will be denoted $p_1(t)$. It is generally more convenient to work with the *fraction* of channels (denoted p) than with the *concentration* of channels (they differ only by a constant, namely the total concentration of channels in the system). The fraction of channels in a given state is also referred to as the *occupancy* of that state.

Observed rate constants

Notice that the observed or *macroscopic time constant* (τ), or its reciprocal the

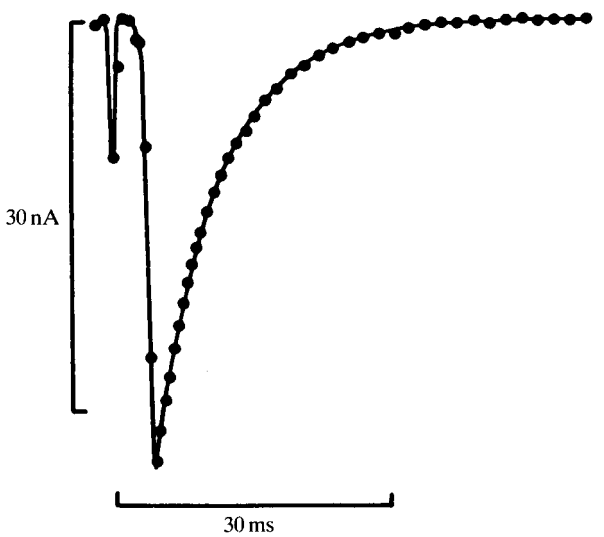


Fig. 1. Endplate current evoked by nerve stimulation (−130 mV, inward current shown downward). The observed current is shown by the filled circles; the continuous line is a fitted single exponential curve with a time constant of 7.1 ms. Reproduced with permission from Colquhoun & Sheridan (1981).

observed rate constant (λ) should be clearly distinguished from the transition rates (k_{12} and k_{21} in this case), and the reaction (mass action) rate constants such as k_{+1}, in the underlying reaction mechanism; this distinction is even more important in more complex cases. Not surprisingly the value of τ depends on *both* of the reaction transition rates; equilibration will be rapid (τ will be small) if *either* the opening rate (k_{21}) is fast or the shutting rate (k_{12}) is fast. Notice that this same expression holds for τ whether the re-equilibration process involves a net increase or a net decrease in the fraction of channels that are open.

The observed time constant can be written in terms of the individual rate constants if we note that the fraction of open channels at equilibrium (after long, effectively infinite time, and therefore denoted $p_1(\infty)$), is

$$p_1(\infty) = k_{21}/(k_{12} + k_{21}) , \tag{2.3}$$

so the fraction of shut channels is $p_2(\infty)=1 - p_1(\infty)$. Hence we can write

$$\tau = p_1(\infty)k_{21}^{-1} = p_2(\infty)k_{12}^{-1} \tag{2.4}$$

Therefore if only a small fraction of channels is open, so $p_2(\infty) \simeq 1$, then, to a good approximation, the observed value of τ gives us directly the value of one of the underlying transition rates, the shutting rate k_{12}. In general, however, the observed τ tells us only about some more or less complicated combination of the transition rates of the mechanism, not about any one of them alone.

So far the argument has shed little light on what is going on at the level of a single channel. The consideration of single channel behaviour will provide a much more concrete mental picture of what underlies the results given so far.

Randomness of single channel behaviour

There are several ways of interpreting the transition rates in terms of individual molecules. These are described in more detail by Colquhoun & Hawkes (1983) in a fairly informal way, and by Horn (1984), Colquhoun & Hawkes (1977, 1982, 1987) in a more formal way. The regular behaviour observed with large aggregates of molecules disappears when we look at a single molecule. The individual molecules behave in an entirely random way. Or, to be more precise, the length of time for which the molecule stays in one of its (supposedly) discrete states is quite random; the properties of the states themselves may be very constant (for example the amplitude of the current while the channel is open is usually very similar from one opening to another of the same channel, and from one channel to another).

The randomness of lifetimes is immediately obvious when looking at single channel records, and it should come as no surprise. Consider, for example, what would happen if the lifetime of individual openings was a fixed constant. During a MEPC a few thousand channels are opened almost simultaneously by a quantum of transmitter (which then quickly disappears); these channels would all stay open for their allotted time and then shut almost simultaneously so thc MEPC would be rectangular in shape. This is not what happens. The nature of the variability will be

discussed below. First two simple interpretations of the transition rates in (2.1) will be given.

Rate constants as transition frequencies

The transition rates have the dimensions of frequency, and they can be interpreted as such. For example in scheme (2.1) k_{12} is the frequency of open $\rightarrow$ shut transitions. Such transitions can, of course, occur only when the channel is open so, to be more precise, k_{12} is the number of shutting transitions per unit open time. The channel is open for a fraction p_1 of the time (this is the single channel equivalent of the *occupancy* defined above), so the overall shutting frequency, the number of shutting transitions per unit time for a single channel, f_s say, is

$$f_s = p_1 k_{12} \tag{2.5}$$

Similarly the frequency of opening transitions for one channel, f_o say, is

$$f_o = p_2 k_{21} \tag{2.6}$$

At equilibrium f_o and f_s will be equal, thus implying the result given in (2.3). It is worth noticing that the rate constants do not tell us anything about how *long* the transition from 'shut' to 'open' takes once it has started, only about how *often* such transitions occur (see, for example, Colquhoun & Ogden, 1987). The transition itself is supposed to be instantaneous; in fact the open-shut transition is unresolvably fast (less than 10 μs, Hamill *et al.*, 1981).

Non-equilibrium conditions. When the macroscopic current is changing with time, as it will, for example after a voltage jump or concentration jump, then the opening and shutting frequencies, f_o and f_s, will change with time and will *not* be equal. The net excess of openings over shuttings per unit time, at time t, is

$$f_o - f_s = p_2(t)k_{21} - p_1(t)k_{12} = k_{21} - p_1(t)(k_{12} + k_{21}) .$$

This is exactly the expression that would be written down for the rate of change of the fraction of open channels, $dp_1(t)/dt$, by application of the conventional macroscopic law of mass action, but now it has been derived by considering the flipping rate of individual channel molecules. The proportionality constant that multiplies $p_1(t)$ in this expression, $(k_{12}+k_{21})$ is precisely the macroscopic observed rate constant, $1/\tau$, as in (2.2). Notice that the unequal frequencies result entirely from the fact that the *occupancies*, p_1 and p_2, are changing with time in (2.5) and (2.6); the fundamental transition rate constants (and hence the observed macroscopic rate constant that depends only on them) are not varying with time, i.e. the membrane potential (or agonist concentration) is held constant at all times following the application the step change (or so, at least, it is assumed in the conventional analyses).

Rate constants and mean lifetime. Another concrete way to think of the meaning of transition rates is to consider their relationship to how long the system stays in a particular state. This length of individual open time is variable, but the *mean* open time is a constant value and it can be shown (see below) that it is simply $1/k_{12}$ for

scheme (2.1). Let us denote the mean lifetime by m; thus the mean open lifetime and the mean shut lifetime (with dimensions of seconds) in (2.1) are, respectively,

$$m_1 = 1/k_{12}$$
$$m_2 = 1/k_{21} \tag{2.7}$$

More generally the *mean lifetime in any individual state can be found by adding up all the values for transition rates that lead out of that state and then taking the reciprocal of this sum.*

By combining the results in (2.7) and (2.3) we find that the equilibrium fraction of open channels can be written in the form

$$p_1(\infty) = \frac{m_1}{m_1 + m_2} \tag{2.8}$$

i.e. it is simply the fraction of the time for which a channel is open, as asserted earlier. Also, from (2.4), the observed macroscopic time constant can be related to the mean lifetimes of the two states as follows:

$$\tau = p_1(\infty)m_2 = p_2(\infty)m_1 = 1/(m_1^{-1} + m_2^{-1}) \, . \tag{2.9}$$

Thus, if most channels are shut ($p_2 \approx 1$) then τ is approximately the mean open lifetime, and if most channels are open ($p_1 \approx 1$) τ is approximately the mean shut lifetime.

Rate constants and probabilities. Clearly the transition rate for shutting, k_{12}, tells us something about the probability that an open channel will shut. But k_{12} has dimensions of s^{-1} so it cannot itself be a probability (which must be dimensionless). In fact the required relationship (approximately)

$$\text{Prob[open channel will shut in a small interval } \Delta t] = k_{12} \, \Delta t \tag{2.10}$$

as long as Δt is small (see, for example, Colquhoun & Hawkes, 1983, for more details).

Numerical example. Suppose that in scheme (2.1) the opening transition rate is k_{21}=250 s^{-1} and the shutting transition rate is k_{12}=1000 s^{-1}. Thus, from (2.3), 20 percent of channels will be open and 80 percent shut at equilibrium (on average). The observed macroscopic time constant for re-equilibration (from equations (2.2), (2.4) or (2.9)) would be τ=1/1250=0.8 ms. The fraction of open channels is not low enough for this to be very close to the mean open lifetime, $1/k_{12}$=1 ms. The mean shut time is $1/k_{21}$=4 ms. From (2.5) and (2.6), each channel would open (and shut) 200 times per second (on average) at equilibrium.

3. The distribution of lifetimes

At this stage we must consider more carefully the nature of the variability of lifetimes. Because lifetimes are random variables their behaviour must be described

by probability distributions, and it is this necessity that makes rather unfamiliar the form in which the information is contained in a single channel record. First a distribution will be derived, and then used to illustrate a discussion of the nature of distributions and density functions.

The distribution of random lifetimes

The first thing to define is what we mean by 'random'. In the present context what we mean is that the probability that an open channel will shut during a short time interval (Δt) is constant, *viz*. $k_{12}\Delta t$ from equation (2.10) regardless of what has gone before (e.g. regardless of how long the channel has already been open). And events that occur in non-overlapping time intervals are independent of each other. In other words what happens in the future depends only on the present state of the system, and not on its past history. Statisticians call processes with this characteristic of 'lack of memory' *homogeneous Markov processes* (named after the Russian mathematician A. A. Markov, 1856-1922, who first studied them).

The conventional derivation of the required distribution starts with equation (2.10); it is given in full by Colquhoun & Hawkes (1983) so it will not be repeated here. Instead an alternative derivation will be given which, if less rigorous, provides greater physical insight (an even simpler version of the following argument is given by Colquhoun & Hawkes, 1983, p.142). This derivation can be regarded as an exploitation of the Markov characteristics of a series of tosses of a coin. The probability of getting 'heads' at each toss is the same, regardless of what has happened before (e.g. regardless of how many consecutive 'heads' have been observed in earlier tosses). The relevant analogy to tossing the coin is the random thermal movements of the channel protein molecule. It is the randomness of thermal motions that underlies the randomness of the lifetimes. The bonds of the protein will be vibrating, bending and stretching, and much of this motion will be very rapid - on a picosecond time scale. One can imagine that each time the open channel molecule 'stretches' there is a chance that all its atoms will get into a position where the molecule has a chance to surmount the energy barrier and flip into the shut conformation. Define p as the probability that the one channel will shut during each 'stretch', so $(1-p)$ is the probability that it will fail to shut. Suppose that this probability is the same at each attempt (each 'stretch') regardless of what has gone before. How many attempts (stretches) will be needed before the channel eventually shuts? The problem is just like asking how many tosses will be needed before the first 'heads' is encountered. The probability of success on the first attempt is p, and the probability of failure on the first attempt but success on the second is the product of the separate probabilities $(1-p)p$. The probabilities can simply be multiplied because the two attempts are supposed to be independent of each other. The probability $P(r)$, of success (i.e. the channel closing) at the rth attempt (i.e. $r-1$ failures followed by a success) is therefore

$$P(r) = (1-p)^{r-1}p, \qquad r = 1, 2, \ldots \infty \tag{3.1}$$

This is called a *geometric distribution* and its mean, μ say, the mean number of attempts before the channel closes, is

$$\mu = \Sigma rP(r) = 1/p \tag{3.2}$$

Now the 'stretching' is on a picosecond time scale, whereas the channel stays open for milliseconds, so p is clearly small, and many 'attempts' will be needed before the channel shuts (*c.f.* coin tossing, for which p=0.5). Suppose further that a 'stretch' occurs every Δt picoseconds (this itself will be random but we shall treat it as though it were constant for the purpose of this argument - doing so does not give a wrong result). The time taken for r attempts is therefore $t=r\Delta t$, and the mean length of time before closure is $\mu\Delta t=\Delta t/p$. Furthermore, from the discussion above we know that the probability of closure during Δt is just $p=k_{12}\Delta t$, so the mean length of time before closure, $\Delta t/p$, is simply $1/k_{12}$, the mean open channel lifetime found before. When r is large we can put $t=r\Delta t$ and write (3.1) in the form

$$\begin{aligned}\text{Prob[channel closes between } t \text{ and } t+\Delta t] = P(r)\\ &\approx k_{12}\Delta t(1-p)^r\\ &= k_{12}\Delta t(1-p)^{k_{12}t/p}\end{aligned} \tag{3.3}$$

Now when Δt is small this geometric expression approaches an exponential form. The 'compound interest formula' states that $(1+1/n)^{nx}$ approaches e^x as n becomes very large (see, for example, Thompson, 1965), where e is the universal constant, the base of natural logarithms, 2.71828.... This formula can be rearranged to show that $(1-p)^{x/p}$ approaches e^{-x} as p becomes very small. Application of this form to (3.3) gives

$$\text{Prob[lifetime between } t \text{ and } t+\Delta t] = k_{12}\Delta t e^{-k_{12}t} \tag{3.4}$$

as long as Δt is small.

In order to express this result as a probability density function (p.d.f.) we must divide by Δt to give the *exponential probability density function*

$$f(t) = k_{12}e^{-k_{12}t} \tag{3.5}$$

This curve is shown in Fig. 2C. Its mean is $1/k_{12}$ (see, for example, Colquhoun, 1971, appendix 1), which proves the result for the mean open lifetime already given in equation (2.7). The area under this curve between 0 and t gives the probability that a lifetime is *less than* the specified t. This is the cumulative exponential distribution, denoted $F(t)$ and is found by integration of (3.5) to be

$$F(t) = 1 - e^{-k_{12}t} \tag{3.6}$$

The last two steps, and the meaning of distributions and p.d.f.s in general will next be explained in a little more detail, before describing further the characteristics of the exponential distribution.

Distributions and density functions

Clearly it makes sense to talk about the probability that a lifetime is *less than* some

specified length, t; this is just the long-run fraction of lifetimes that are less than t. It is referred to as the *cumulative distribution*, or *distribution function*, and is usually denoted $F(t)$. It starts at zero for t=0 and rises towards 1 for long enough times. Thus

$$\text{Prob[lifetime} < t] \equiv F(t) \; . \tag{3.7}$$

For the exponential distribution this is $1-e^{-k_{12}t}$ as shown in (3.6). Clearly it also makes sense to talk about the probability that a lifetime lies in the interval Δt between two specified time values, t and $t+\Delta t$. Again this is the fraction of lifetimes that lie in this range in the long run, and it is given, in the above notation by

$$\text{Prob[lifetime between } t \text{ and } t + \Delta t] = F(t + \Delta t) - F(t) \; . \tag{3.8}$$

Our experimental estimate of this is what goes into the histogram bin that stretches from t to $t+\Delta t$ (see Chapter 6).

What does *not* make sense is to ask what is the probability of the lifetime being t, e.g. the probability that it is *exactly* 5 ms, as opposed to 4.999 ms, or 5.001 ms say (we are dealing with a theoretical distribution so the question of how precisely values can be estimated experimentally does not arise). When Δt is made small then (3.4) and (3.8) approach zero; there is virtually no chance of seeing an *exactly* specified value. It is for this reason that we have to introduce the idea of a *probability density function* (p.d.f.). When Δt approaches zero, so t and $t+\Delta t$ converge on a single value, then (3.4) and (3.8) also approach zero. However the ratio of these two quantities does not. The p.d.f. is defined as this ratio, and is usually denoted $f(t)$. This ratio is

$$f(t) = \frac{F(t + \Delta t) - F(t)}{\Delta t} \longrightarrow \frac{\mathrm{d}F(t)}{\mathrm{d}t} \tag{3.9}$$

For the exponential distribution this gives the result presented in equation (3.5) above. When Δt is very small this function becomes $\mathrm{d}F(t)/\mathrm{d}t$, the first derivative of the distribution function. *The p.d.f. is clearly not itself a probability (its dimensions are* s^{-1}*), but it is a function such that the area under the curve (which is dimensionless) between any specified time values is the probability of observing a lifetime between these values.*

The form of the variability is completely specified by either the cumulative distribution or the p.d.f., but the latter should normally be used (see comments in chapter on fitting of data). We now return to explore the properties of the exponential distribution in more detail.

Some properties of the exponential distribution

The exponential p.d.f. given in (3.5) has a rather unusual shape compared with other p.d.f.s that may be more familiar. For example Fig. 2A shows the symmetrical bell-shaped p.d.f. of the Gaussian ('normal') distribution.

Symmetry ensures that the *mode* (peak, or 'most frequent value'), the *median* (below which 50 percent of values lie) and the *mean* are all the same for the Gaussian. Fig. 2B shows a lognormal p.d.f. (for which the log of the variable is Gaussian); the

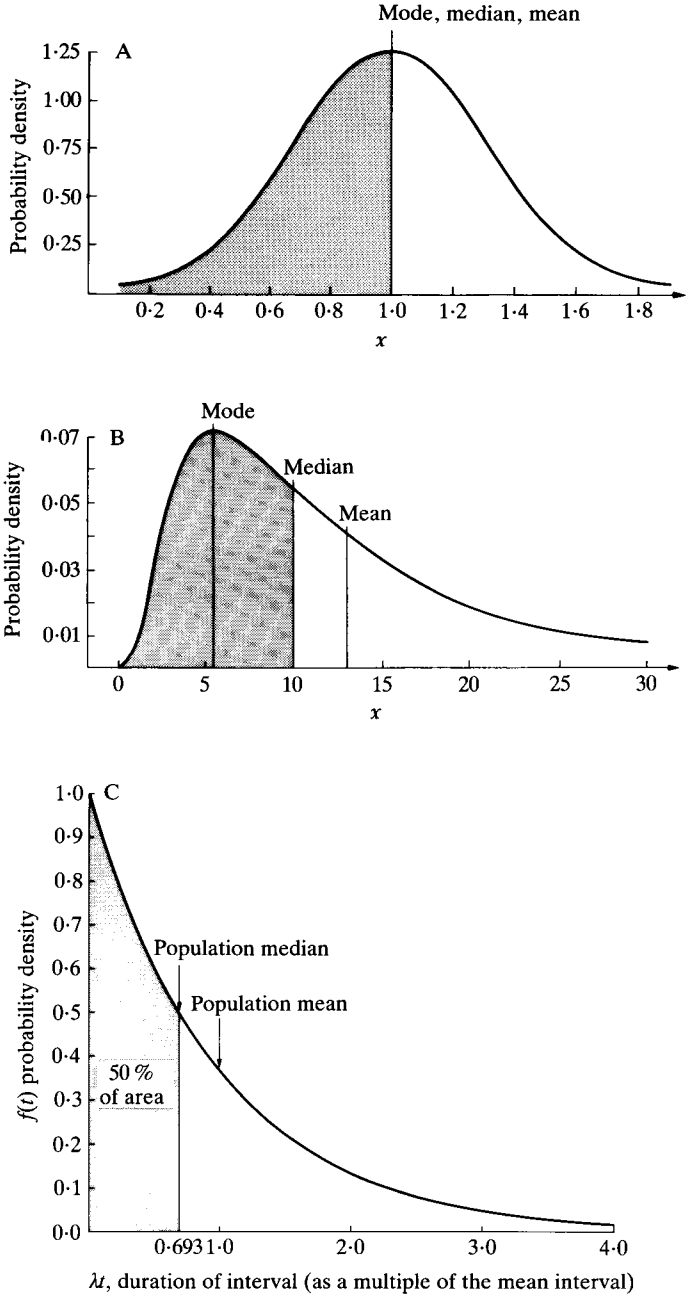


Fig. 2. (A) The p.d.f. for a Gaussian (or 'normal') distribution. This example has a mean of 1.0 and a standard deviation of 0.32. (B) The p.d.f. for a lognormal distribution (a distribution such that log x is Gaussian). (C) The p.d.f. for the exponential distribution (eqn (3.5)). The abscissa shows the value of the variable (e.g. open lifetime) expressed as a multiple of its mean.

positive skew of this p.d.f. means that mean > median > mode. Fig. 2C shows the exponential p.d.f. of lifetimes which is seen to be an extreme case of a positively skewed distribution. The modal lifetime is zero. The mean of the exponential distribution is seen to be what we would call the time constant if the exponential curve in Fig. 2C were describing a decaying current rather than a p.d.f. If we call the mean lifetime τ, then we can write the exponential p.d.f., (3.5), in an alternative form as

$$f(t) = \tau^{-1}e^{-t/\tau} \tag{3.10}$$

The median of this distribution is 0.693τ (see below). Notice that in our simple example $\tau=1/k_{12}$ is *not* the same as the time constant, $1/(k_{12}+k_{21})$, from macroscopic measurements (they will be similar only if most channels are shut, as shown above). The single channel measurement is simpler in that it gives directly one of the underlying reaction transition rates (k_{12}) whereas the macroscopic measurement gives only a combination of them.

An exponential current decay and an exponential p.d.f. are quite different sorts of things, but it is not a coincidence that they both have the same shape. Fig. 3 shows a simulation of what happens during the decay of an MEPC. Many channels (five of which are shown in Fig. 3) are opened almost simultaneously by the transmitter, and each of them stays open for an exponentially-distributed length of time before closing.

Once closed they will not re-open because the transmitter concentration falls rapidly to zero - this means that opening rate is zero, so in this particular case, though not in general, the macroscopic time constant corresponds to the mean open lifetime. At any particular moment, the total current that flows is proportional to the total number of open channels, and this is shown in the lower part of Fig. 3. It shows an exponential decay of the current. This is expected because the fraction of channels that are open at t will be those that have a lifetime longer than t, i.e. $1-F(t)=e^{-t/\tau}$ (see equation (3.6)). The relationship between the smooth macroscopic behaviour and the underlying random behaviour of single channels should now be clear.

The median lifetime for an exponential p.d.f., 0.693τ, is defined such that 50 percent of lifetimes are shorter than the median and 50 percent are longer. This is the single-channel equivalent of the *half-time* for the decay of the MEPC. The proportion of lifetimes that a shorter than the mean is 63.2 percent, so 36.8 percent of intervals are longer-than-average lifetimes. However, although longer-than- average lifetimes are in a minority, their length is such that they actually occupy a greater proportion (75 percent) of the time than is occupied by the more numerous shorter-than-average lifetimes (see Colquhoun, 1971, for details). This fact means that if we stick a pin into the record at random we will, in the long run, tend to pick out longer-than average intervals. In fact those picked out will have, in the long run, twice the mean lifetime (a phenomenon known as length-biased sampling). It is this rather curious property that is responsible for some of the unexpected characteristics of random lifetimes.

The waiting time paradox. The property of length-biased sampling explains, for example, why we get the same macroscopic time constant whether the openings of

channels are all lined up at t=0 (as in the MEPC illustrated in Fig. 3), or whether the channel is already at equilibrium with agonist before agonist is suddenly removed at t=0. In the latter case (but not the former) every channel that was open at t=0 (those whose shutting is subsequently followed) will already have been open for some time so one might think that half of their average lifetime had already been 'used up'. In a sense it has, but since the particular channels that were open at t=0 had a twice-normal lifetime because of length-biased sampling, the amount of time they have left to stay open is just on average, the overall mean open time, just as in the case of the MEPC.

The same sort of phenomenon explains why the distribution of the duration of the shut time that precedes the first opening after a voltage jump (the first latency) will be the same as the distribution of any other shut time. This is true, at least, for the simple mechanism discussed above, which has only one sort of shut state (it is not, however, always true - see discussion of correlations in section 6).

This waiting-time paradox, and other unexpected characteristics of random

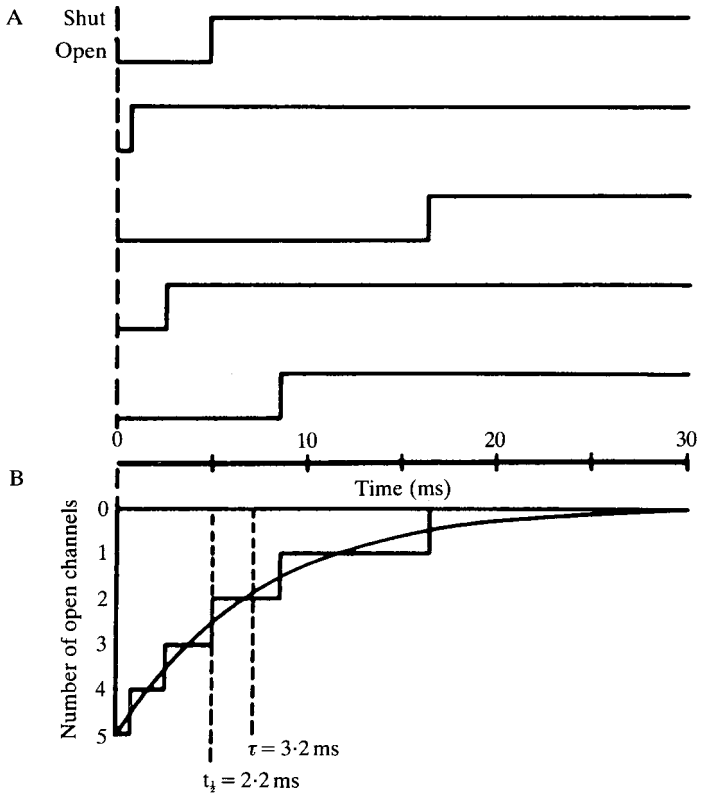


Fig. 3. Simulation of the decay of an MEPC. The upper part shows five individual channels. They have exponentially distributed lifetimes with a mean of 3.2 ms (and a median of 2.2 ms). The lower part shows the sum of these five channels with a smooth exponential curve superimposed on it. The curve has a time constant of 3.2 ms and a half-decay time of 2.2 ms. Reproduced with permission from Colquhoun & Hawkes (1983).

lifetimes are discussed in greater detail by Colquhoun (1971) and Colquhoun & Hawkes (1983).

The sum of several intervals - the idea of convolution

There are many occasions when it is necessary to consider not just a single interval, but the sum of several intervals. For example, when there are several shut states that intercommunicate directly (as in mechanism (4.1) below), a single observed shut period will generally consist of several oscillations between one shut state and another - the duration of the observed shut period will be the sum of the durations of all these (exponentially-distributed) sojourns. Similarly the duration of a *burst* of openings (e.g. section 5) will be the sum of each of the open and shut times that make up the burst. Another example occurs when the time course of synaptic currents is considered (see section 7 below); it is then important to consider the sum of the latency until the first channel activation plus the duration of the activation.

The distribution of the sum of several random intervals can be found by a method known as *convolution.* This method is central to much of single channel analysis, so the technique will be introduced here by the simplest case of its use.

An example of convolution: the distribution of the sum of two intervals. We shall derive the distribution of the sum of two exponentially distributed intervals. In the context of the simplest mechanism (2.1), we can derive the distribution of the sum of an open time and a shut time, i.e. the time from the start of one opening to the start of the next. Consider an individual open time; it is an exponentially- distributed variable, with mean $1/k_{12}$; its p.d.f. is

$$f_1(t) = k_{12}e^{-k_{12}t} \tag{3.11}$$

Similarly the mean shut time is $1/k_{21}$, and its p.d.f. is

$$f_2(t) = k_{12}e^{-k_{21}t} \tag{3.12}$$

Suppose that we now make a new variable by adding an open time and a shut time. These new values will, of course, be variable; their *mean* value will obviously be the sum of the separate means, $(1/k_{12})+(1/k_{21})$, but we wish to know what sort of distribution (p.d.f.) describes these new values. The p.d.f. of this variable, denoted $f(t)$ say, can be found by noting that any *specified* duration, t, of the sum, can be made in all sorts of different ways. If we denote the length of the open time as t_1, and the length of the shut time as t_2, then the total length is $t=t_1+t_2$. Now if the open time happens to have a length $t_1=u$, then, in order for the total length to be t, the length of the shut time must be $t-u$. And, for any fixed value, t, of the total length, u can have *any* value from 0 up to t (the former corresponds to the case where the total length consists entirely of t_2, the latter to the case where it consists entirely of t_1). The probability density that $t_1=u$ and $t_2=t-u$ can be found by multiplying the separate probability densities (because these two events are independent), i.e. from (3.11) and (3.12) it is $f_1(u)f_2(t-u)$. Since any value of u

(from 0 to t) will do, we must now add these probabilities for each possible value of u: since u is a continuous variable this addition takes the form of an integration, and the result is

$$f(t) = \int_{u=0}^{u=t} f_1(u) f_2(t-u)\, \mathrm{d}u \tag{3.13}$$

and, in this case the integral can easily be evaluated explicitly to give (as long as k_{12} and k_{21} are different)

$$f(t) = \frac{k_{12}k_{21}}{k_{21}-k_{12}} (\mathrm{e}^{-k_{12}t} - \mathrm{e}^{-k_{21}t}) . \tag{3.14}$$

This is plotted in Fig. 4.

Notice that, unlike the simple exponential distribution, this distribution goes through a maximum, at

$$t_{\max} = \frac{\ln(k_{21}/k_{12})}{k_{21} - k_{12}} . \tag{3.15}$$

In other words the most common (modal) values are no longer the shortest values, but values around $t_{\max}$. This is not surprising because it is unlikely that t_1 and t_2 will *both* be very short. The distribution in (3.14) consists of two exponential terms with opposite signs; one exponential represents the rising phase of the distribution, and one the decay phase. Notice that (3.14) is completely unchanged if k_{12} and k_{21} are interchanged: whichever is the faster represents the rising phase, and the slower represents the decay phase. In other words the distribution of the sum of two intervals does not depend on whether the 'short one' or the 'long one' comes first.

Equations with the form of (3.13) are known as *convolution integrals*. Such equations occur whenever we consider the distribution of the sum of random variables, and are the basis for obtaining things like the distribution of burst lengths, though in cases more complex than this it is necessary to use Laplace transforms and matrix methods to obtain useful results (e.g. see Colquhoun & Hawkes, 1982). (Incidentally, convolution integrals also occur in single channel work in a rather different context: in linear systems such as electronic filters, the output of the system can be found by convolving the input with the impulse response function for the system, i.e. the input is represented as a series of impulse responses, the outputs for which superimpose linearly; see, for example, Chapter 6, and Colquhoun & Sigworth, 1983.)

Summary of results so far

The length of time for which the channel stays in an individual state of the system (the lifetime of this state) is a random variable. The variability is described by an

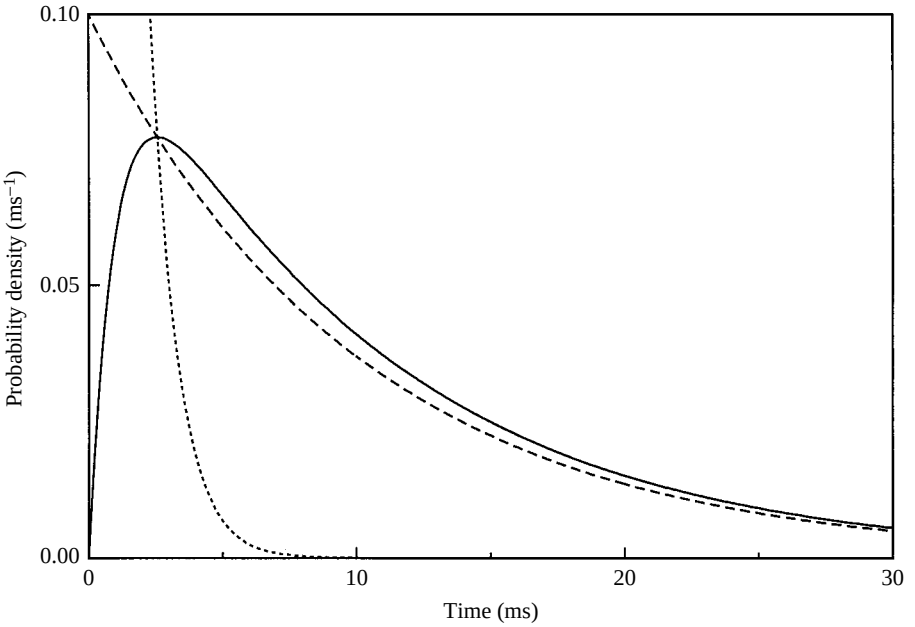


Fig. 4. Plot of two exponential distributions with rate constants k_{12}=1 ms^{-1} (mean lifetime=1 ms) (dotted curve), and k_{21}=0.1 ms^{-1} (mean lifetime=10 ms) (dashed curve). The former curve goes off-scale (intercept on the *y* axis is 1.0 at *t*=0). The solid line shows the convolution of these two distributions, from (3.14), i.e. the distribution of the sum of a '1 ms' amd a '10 ms' interval.

exponential p.d.f., $f(t)=\tau^{-1}e^{-t/\tau}$, the mean channel lifetime, τ, being the reciprocal of the sum of all transition rates for leaving the state in question. The exponential distribution of lifetimes is what underlies the exponential reequilibration seen in macroscopic measurements. The relationship between the observed time constants and the reaction transition rates in the underlying mechanism is indirect, but will usually (not always) be simpler for single channel measurements than for macroscopic measurements.

So far the only concrete example that has been considered, scheme (2.1), was simple because it had only two states, and because these two states could be distinguished on an experimental record. We must now consider what happens when there are more than two states, and, in particular, when not all of the states can be distinguished from one another by looking at the experimental record.

4. Some more realistic mechanisms: rapidly-equilibrating steps

We shall consider two simple mechanisms, each of which has two shut states and one open state.

An agonist activated channel

Consider, for example, a mechanism in which an agonist (A) binds to a receptor (R), following which an isomerization to the active state (i.e. the open channel, R*) may occur. This scheme, first proposed by Castillo & Katz (1957), can be written

(4.1)

The rate constants are denoted by the symbols on the arrows, and $K_A=k_{-1}/k_{+1}$, is the equilibrium constant for the initial binding step. In fact, for many fast transmitters, more than one agonist molecule must be bound to open the channel efficiently, but the simple case in (4.1) will suffice to illustrate the principles. It has three states, and the lifetime in each of these states is expected to be exponentially distributed with, according to the rule given above, the mean lifetimes for states 1, 2 and 3 being

$$m_1 = 1/\alpha$$
$$m_2 = 1/(\beta + k_{-1})$$
$$m_3 = 1/k_{+1}x_A \qquad (4.2)$$

respectively (where x_A is the free concentration of the agonist). The fraction of channels that are open at equilibrium, $p_1(\infty)$, is

$$p_1(\infty) = \frac{(x_A/K_A)(\beta/\alpha)}{1 + (x_A/K_A)(1 + \beta/\alpha)} \qquad (4.3)$$

Now we can, in principle, measure the distribution of open lifetimes from the experimental record (but see discussion of bursts, and of missed events, below); the mean open time will provide an estimate of α directly. However a new problem arises when we consider shut times, because the two sorts of shut state (states 2 and 3) cannot be distinguished on the record (again see below). We therefore cannot say which of them a shut channel is in, so we cannot measure separately the mean lifetimes of states 2 and 3 in order to exploit directly the relationships in (4.2). This problem will be dealt with in §5.

A channel block mechanism

Another example with two shut states and one open state (but connected differently)

is provided by the case where a channel, once open, can be subsequently plugged by an antagonist molecule in solution. This can be written

(4.4)

By the same rule as before the mean lifetimes of sojourns in open, blocked and shut states will be, respectively,

$$m_1 = 1/(\alpha + k_{+B}x_B)$$
$$m_2 = 1/\, k_{-B}$$
$$m_3 = 1/\beta', \qquad (4.5)$$

where x_B is the free concentration of the antagonist. Once again, the two non-conducting states (shut and blocked) cannot be distinguished by inspection of the experimental record.

Before considering these two examples in a general way, it will be helpful to consider what happens when several states equilibrate with each other rapidly.

Pooling states that equilibrate rapidly

The analysis becomes simpler (but also less informative) if some states interconvert so rapidly that they behave kinetically like a single state.

Rapid binding. Consider, for example, what would happen if the binding and dissociation of agonist in scheme (4.1) were very rapid compared with the subsequent opening and shutting of the occupied channel. (This is probably not true for the nicotinic acetylcholine receptor, but it makes an interesting example.) The two shut states then appear to merge into one; this may be indicated by enclosing both in one box, and writing the mechanism thus

(4.6)

This now looks formally just like the simple two-state case discussed first (equation (2.1)), and can be analysed as such. (If the channel being blocked in scheme (4.4) were an agonist activated channel then an approximation of this sort would be implicit in (4.4), which has been written with only one shut (as opposed to blocked) state.)

The channel shutting rate constant is α, just as in (4.1), but the opening rate

constant needs some consideration. The real rate constant for opening is β in (4.1), the rate constant for AR→AR* transition. However, the compound 'shut' state in (4.6) is sometimes occupied (AR) and sometimes not (R), but the opening transition is possible *only* when it is occupied. Thus the *effective opening rate constant*, β′, for transition from the compound shut state in (4.6) to the open state, must be defined as the true opening rate constant, β, multiplied by the fraction of time for which shut receptors are occupied (this fraction, and hence β′, will increase with agonist concentration). Because the binding reaction has been supposed to be rapid this fraction will essentially not vary with time (it will always have its equilibrium value), so β′ will not vary with time, and the reaction scheme is just like scheme (2.1). Hence, from results in (2.2) to (2.9), the open lifetimes and the shut lifetimes will both have simple exponential distributions, with means $1/\alpha$ and $1/\beta'$ respectively. The macroscopic time constant will be $\tau=1/(\alpha+\beta')$, which will approximate to the mean shut time if most channels are open, or to the mean open time if most channels are shut. The equilibrium fraction of open channels will be $p_1(\infty)=\beta'/(\alpha+\beta')$, which is another way of writing the result in (4.3). There will be $p_1(\infty)\alpha$ channel shuttings (and openings) per second at equilibrium.

How fast is fast? The answer is that it is all relative. An interesting example is provided by a scheme like (4.4) in which the blocked state is very long- lived. An exactly similar example would be provided by a model in which the open state could lead to a very long-lived desensitized state (AD say). In such a case the shut ⇌ open equilibrium, while possibly slower than the binding reaction, might nevertheless be much faster than the desensitization reaction. In this case *all* of the states in (4.1) might be effectively pooled into a single 'active' (i.e. non-desensitized) state to give

(4.7)

('active')

Here k_{-D} is the rate constant for recovery from desensitization, and k'_{+D} is the *effective* rate constant for moving into the desensitized state (i.e. the true AR*→AD rate constant, multiplied by the equilibrium fraction of active channels that are open, and hence available to be desensitized). Again this looks just like the original two state scheme in (2.1), and the results derived for that scheme can be applied. For example the macroscopic rate constant for the desensitization will be $\tau=1/(k'_{+D}+k_{-D})$. The mean lifetime of the active state (each sojourn in which will consist of many openings) will be $1/k'_{+D}$, and the mean lifetime of the desensitized state will be $1/k_{-D}$. In fact long silent desensitized periods are observed in single channel records at high agonist concentrations (Sakmann, Patlak & Neher, 1980; Colquhoun, Ogden & Cachelin, 1986; see also Chapter 6). It often seems to be supposed that if, say, 10

second silent desensitized periods appear in the single channel record then we should see that macroscopic desensitization (at the same agonist concentration) develops with a similar time constant. However the argument just presented shows that, insofar as the desensitized periods are much longer than the non-desensitized active periods, then the macroscopic time constant should be closer to the mean length of the periods of activity than to the mean lengths of the silent desensitized periods.

5. Analysis of mechanisms with three states

Under the usual assumptions (see above) we would expect macroscopic observations on any mechanism with three states to reequilibrate along a time course described by the sum of two exponentials with different time constants (τ_s and τ_f say, for the slower and faster values respectively). In the cases just discussed, where some states equilibrate very rapidly with each other, one of the time constants would be so fast as to be undetectable, so we would appear to have only two states. We must now consider the case when this does *not* happen.

A channel block example

Fig. 5 shows an example where two exponentials are clearly visible: it is an endplate current recorded in the presence of a channel blocking agent, gallamine, for which τ_s =28.1 ms and τ_f=1.37 ms. Noise spectra also show two components and the results can be accounted for by a simple block mechanism in (4.4), as described by Colquhoun & Sheridan (1981).

As in the simplest case the macroscopic time constants (τ_s, τ_f) or their reciprocals, the macroscopic rate constants (λ_s, λ_f), are indirectly related to all of the transition

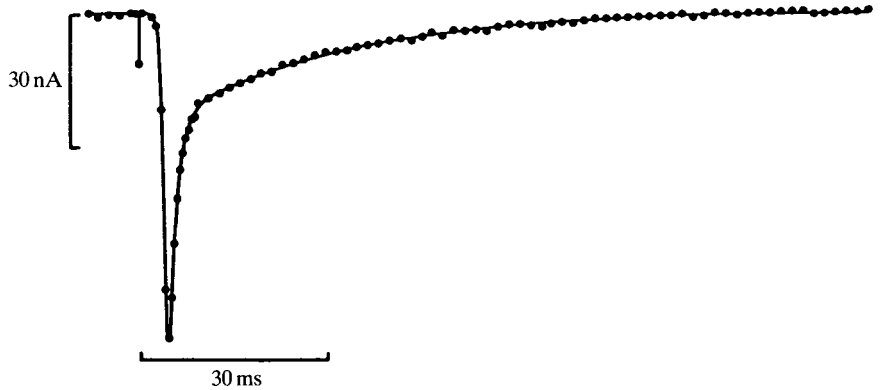


Fig. 5. An evoked endplate current as in Fig. 1 but recorded in the presence of a channel blocking agent (gallamine 5 μM). The current is shown by the closed circles the fitted double exponential curve has time constants τ_f=1.37 ms, τ_s=28.1 ms. Reproduced with permission from Colquhoun & Sheridan (1981).

rates in the underlying mechanism. The macroscopic rate constants in this case are found by solving a quadratic equation,

$$\lambda^2 + b\lambda + c = 0\ . \tag{5.1}$$

The well-known solution of this quadratic is

$$\lambda_s,\ \lambda_f = 0.5\left(-b \pm \sqrt{b^2 - 4c}\right). \tag{5.2}$$

A less well-known alternative is

$$\lambda_s,\ \lambda_f = \frac{2c}{-b \mp \sqrt{b^2 - 4c}}, \tag{5.3}$$

where

$$-b = \lambda_s + \lambda_f \tag{5.4}$$

and

$$c = \lambda_s\lambda_f\ . \tag{5.5}$$

At this point it will be useful to point out that when one of the rate constants is much bigger than the other (say $\lambda_f \gg \lambda_s$, where the subscripts denote 'fast' and 'slow'), i.e. when $b^2 \gg c$, then it follows from (5.2) and (5.4) that the faster rate constant, λ_f, is approximately

$$\lambda_f \approx -b \tag{5.6}$$

and, from (5.3) and (5.5), the slower rate constant, λ_s, is approximately

$$\lambda_s \approx -c/b \tag{5.7}$$

In this case the coefficients, b and c, are given by

$$-b = \alpha + \beta' + k_{+B}x_B + k_{-B}\ , \tag{5.8}$$

$$c = \alpha k_{-B}\left[1 + \frac{\beta'}{\alpha}\left(1 + \frac{x_B}{K_B}\right)\right], \tag{5.9}$$

and $K_B = k_{-B}/k_{+B}$ is the equilibrium constant for binding of the blocking agent (concentration x_B) to the open channel. More generally when there are n states the $n-1$ macroscopic rate constants will be solutions of a polynomial of degree $n-1$, and their sum will be equal to the sum of all the underlying reaction transition rates, as in (5.4). A third parameter, the relative amplitudes of the fast and slow components, is needed to describe completely results such as those in Fig. 5. This too is related (in a still more complex way) to the reaction transition rates; it can be calculated by methods such as those described by Colquhoun & Hawkes (1977). It is a very

important stage in the testing of a putative mechanism to ensure that not only the time constants, but also their amplitudes, are as predicted by the mechanism.

In order to test the fit of the channel block mechanism, (4.4), to experimental data we can, for example, see whether λ_s and λ_f (or, more conveniently, $\lambda_s+\lambda_f$ and $\lambda_s\lambda_f$), and the relative amplitude of the two components, vary with the concentration of blocker (x_B) and the concentration of agonist (which changes β' - see above) in the predicted way. If it does then values for the underlying transition rates, α, k_{-B} etc. can be extracted; in this process it is often useful to use approximations to the exact equations (such as (5.6) and (5.7)), and various procedures have been described (see, for example, Adams, 1976; Adams & Sakmann, 1978; Colquhoun, Dreyer & Sheridan, 1979; Colquhoun & Sheridan, 1981).

A simple agonist example

Similar arguments apply to the simple agonist mechanism in (4.1). Again two macroscopic rate constants are predicted, their values being found from the quadratic solution (5.2), but in this case the coefficients are given (e.g. Colquhoun & Hawkes, 1977) by

$$-b = \lambda_s + \lambda_f = \alpha + \beta + k_{+1}x_A + k_{-1} \tag{5.10}$$

$$c = \lambda_s\lambda_f = \alpha k_{-1}\left[1 + \frac{x_A}{K_A}\frac{(\alpha+\beta)}{\alpha}\right]$$

$$= \alpha k_{-1} + \alpha k_{+1}x_A + \beta k_{+1}x_A \tag{5.11}$$

where K_A is the microscopic equilibrium constant for the binding reaction, i.e. k_{-1}/k_{+1}. The problem with trying to use this result is that it is *not* generally possible to see experimentally the predicted two components in macroscopic responses to agonists. Usually, as in Fig. 1, a single exponential is a good fit. It is for this reason that it was postulated that agonist binding is very fast, as described in (4.6). However this is not the only possible explanation (and is probably not the correct explanation). In order to get further, the greater discriminating power of single channel measurements are needed.

Single channel results with more than two states

In practice it is usually observed, for virtually every type of channel, that the p.d.f. required to fit the distribution of shut times has more than one exponential component. For example Colquhoun & Sakmann (1985) found that three components were needed for the nicotinic channel even at low agonist concentrations (see Fig. 10 in Chapter 6). Under the usual assumptions, the number of components in this distribution provides a (minimum) estimate of the number of shut states (see Colquhoun & Hawkes, 1982). Likewise the number of components in the open time distribution indicates the (minimum) number of open states. These results are rather more informative than for macroscopic measurements for which the number of components (minus one) indicates the total number of states, but doesn't indicate how many of these are open states.

Thus both the examples above, the agonist mechanism in (4.1) and the channel block mechanism in (4.4), predict a double exponential shut time p.d.f. and a single exponential open time p.d.f. The open time p.d.f. will therefore be

$$f(t) = \tau^{-1}e^{-t/\tau} \tag{5.12}$$

where τ is the mean open time given in (4.2) and (4.5) for the two models (notice that this is decreased by increasing the blocker concentration for the channel block case). The shut time p.d.f. should have the double-exponential form (see Chapter 6)

$$f(t) = a_s\tau_s^{-1}e^{-t/\tau_s} + a_f\tau_f^{-1}e^{-t/\tau_f}, \tag{5.13}$$

where a_s and a_f are the relative areas under the p.d.f. accounted for by the slow and fast components respectively, and τ_s and τ_f are the observed 'time constants', or 'means', of the two components. We may also refer to their reciprocals, the observed rate constants for the shut time distributions, $\lambda_s=1/\tau_s$ and $\lambda_f=1/\tau_f$. These time constants are not, of course, the same as those found for macroscopic measurements. We must, therefore, now enquire how they are related to the underlying reaction transition rates.

Shut times for channel block. This case is particularly simple because no direct transition is possible between the two sorts of shut state in (4.4). Every shutting of the channel must, therefore, consist of *either* a single sojourn in the shut state (state 3), *or* a single sojourn in the blocked state (state 2). The two time constants in this case are therefore simply the mean lifetimes of these two states, $m_3=1/\beta'$ and $m_2=1/k_{-B}$ (the mean lifetime of a blockage). And the relative areas are determined simply by the relative frequency of sojourns in each of these states, i.e. on the relative values of α and $k_{+B}x_B$. These results are a great deal simpler than those for macroscopic measurements given in (5.8)-(5.9); the values of two transition rates can be found directly from τ_s and τ_f.

Shut times for the agonist mechanism. The scheme in (4.1) is a bit more complicated than that for channel block; the reason for this is that the two shut states intercommunicate directly. Thus an observed shut period *might* consist of a single sojourn in AR (state 2) followed by reopening of the channel, but it might also consist of a variable number of transitions from AR to R and back before another opening occurred (both states are shut so these transitions would not be visible on the experimental record). This complication means that we once again have to resort to solving a quadratic, as in (5.2), to predict the observed time constants for the shut time distribution. One way in which the distribution of shut times can be derived, for this particular mechanism, is given in appendix 1. By use of the matrix methods given by Colquhoun & Hawkes (1982), a general expression for the shut time distribution can be obtained that holds for *any* mechanism. In this case the observed rate constants are given by (e.g. appendix 1, and Colquhoun & Hawkes, 1981) by solving the quadratic, as in (5.1)-(5.7),

$$-b = \lambda_s + \lambda_f = \beta + k_{+1}x_A + k_{-1} \tag{5.14}$$

$$c = \lambda_s\lambda_f = \beta k_{+1}x_A \, . \tag{5.15}$$

This result, although more complex than for the channel block mechanism, is still a good deal simpler than the analogous result for macroscopic measurements given in (5.10) and (5.11). For example, the rate constant α does not appear at all here (only those transition rates that lead *away from* shut states appear), and the expressions are simpler. Nevertheless, as in the macroscopic case, the observed time constants are not *directly* related to individual reaction transition rates or to the mean lifetimes of states.

Although this statement is generally true, we can often find good approximations that lend a simple physical significance to the time constants. If, for example, we observe that one rate is very much faster than the other ($\lambda_f \gg \lambda_s$) then (5.14) will give a good approximation to λ_f; furthermore when the agonist concentration (x_A) is very low the term $k_{+1}x_A$ will be negligible, so under these conditions

$$\tau_f = 1/\lambda_f \approx \frac{1}{\beta + k_{-1}} \, , \tag{5.16}$$

and this is just the mean lifetime of a single sojourn in the AR state, as was pointed out in (4.2). Note, though, that while this is *exactly* the mean lifetime of AR, it is only *approximately* the fast time constant of the shut time distribution. The physical significance of this is that short shut periods (gaps) consisting of a single sojourn in AR are predicted to appear in between openings of the channel as a result of AR*→AR→AR* . . . transitions. If, while in AR, the agonist dissociates (AR→R), rather than the channel reopening (AR→AR*), then a much longer shut period will ensue, which will contribute to the slow component of the distribution of shut times. Thus the openings will appear to occur in rapid bursts.

Bursts of channel openings

Whenever the shut time distribution has one time constant that is much shorter than another the experimental record will contain short shut periods and some much longer ones (see, for example, the shut time distribution shown in Fig. 10 of Chapter 6). In this case several openings may occur, each opening separated from the next by a short shut period, before a long shut period occurs. The openings, will therefore appear to be grouped together in *bursts* of openings, each burst being separated from the next by a relatively long silent period. Examples of such bursts are shown, for example, by Neher & Steinbach (1978), Ogden, Siegelbaum & Colquhoun (1981), and Ogden & Colquhoun (1985) for channel blockers and by Colquhoun & Sakmann (1981, 1983, 1985) and Sine & Steinbach (1985) for agonists. Some bursts are illustrated in Fig. 6.

It may simplify the analysis considerably if the experimental record can be divided up, without too much ambiguity, into such bursts. A major advantage of doing this is that we can usually be sure that all the openings within one burst originate from the same individual channel, so the characteristics of shut periods

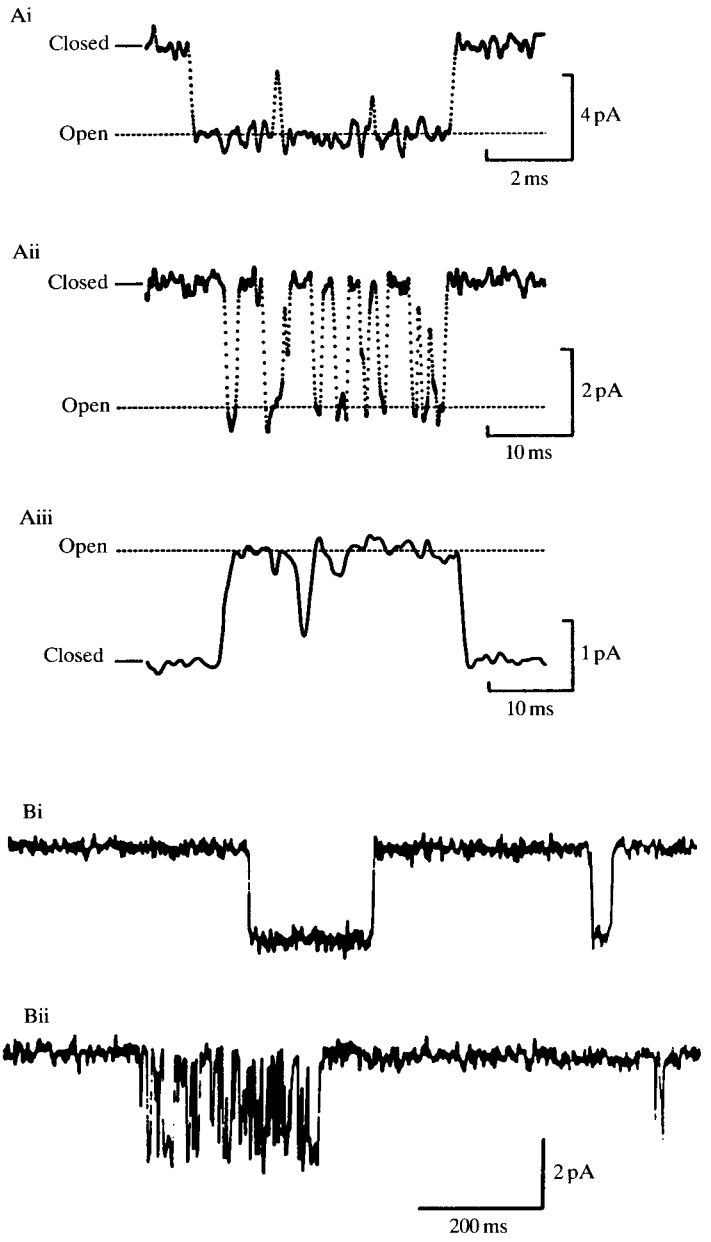


Fig. 6. (A) Burst-like appearance of single channel currents in different transmitter-activated ion channels. (Ai) Acetylcholine-activated single channel current recorded from the frog endplate. Downward deflection of the trace represents inward current. (Aii) Acetylcholine-activated single channel current recorded from a sinoatrial node cell of rabbit heart. Downward deflection represents inward K^+ current. (Aiii) Glycine-activated single channel current in soma membrane of a cultured spinal cord cell. Upward deflection of the trace corresponds to an outward current reflecting Cl^- influx into the neuron. Reproduced with permission from Colquhoun & Sakmann (1983). (B) Bursts produced by rapid channel block. (Bi) Acetylcholine activated channels at frog end-plate. (Bii) The same in the presence of benzocaine (200 μM). Reproduced from Ogden, Siegelbaum & Colquhoun (1981).

within bursts can tell us something about the channel. On the other hand individual well-separated bursts may well originate from different channels so, in the absence of knowledge of how many channels there are in the membrane patch, it is impossible to interpret the lengths of gaps *between* bursts in any useful way. Another advantage of looking at the properties of bursts of openings is that the interpretation of the results may be simplified; long-lived shut states are, by definition, excluded from the bursts so we need consider a smaller subset of states than would otherwise be necessary.

Bursts with channel blockers

The appearance of bursts in the presence of a channel blocking agent will depend on how long the blockages last. If the blockages last for a long time (e.g. seconds, i.e. k_{-B} is small) the blocked periods will not be distinguishable from any other shut time, and the record will not look obviously bursty. At the other extreme, if blockages are very brief (i.e. k_{-B} is very large) so that they are mostly not resolvable, it will appear (incorrectly) that the single channel amplitude is depressed; this happens partly because such blockers will have a low affinity for the receptors, and so will be used in concentrations that make the openings very short (see (4.5)), as well as the blockages being short (see Ogden & Colquhoun, 1985, for an example).

Clear bursts will be seen in the presence of channel blockers when the blockages are of a duration that is comparable with the channel open times, so blockages appear as brief interruptions of a normal channel activation. Each individual opening will be shorter in the presence of the blocking agent, as shown in (4.5), and the predicted linear dependence of the reciprocal open time on blocker concentration provides a test of the model (and, if the test is passed, an estimate of the association rate constant for block of an open channel, k_{+B}). An example of the linear dependence of the reciprocal mean open time on blocker concentration is shown in Fig. 7 (filled circles).

The line is straight as predicted by (4.5) so the slope provides an estimate of the association rate constant for the blocking reaction ($k_{+B}=3.9\times10^{7}$ M^{-1} s^{-1} in Fig. 7). In this case the mean open times had been corrected for the fact that the brief interruptions, which the agonist (suberyldicholine) shows even in the absence of block, are only partially resolved. The intercept should therefore give an estimate of α. Another approach would be to ignore these spontaneous shuttings and look at the shortening of whole elementary event (the burst of openings produced by a single activation of the channel) by channel blocking; this should also give a slope of k_{+B} (Ogden & Colquhoun, 1985).

Fallacies in the interpretation of channel block

The shortening of the mean open time by a channel blocker might be regarded as resulting from openings being ‘cut short’ by the insertion of a blocking molecule into the open channel. This view seems consistent with the fact that the mechanism (4.4) predicts that the mean value of total open time in a burst is unaltered by the presence

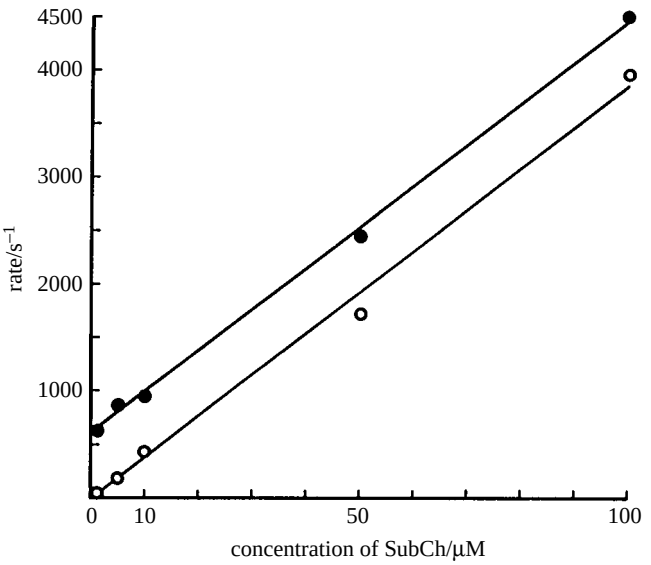


Fig. 7. Block of suberyldicholine-activated end-plate channels by the agonist itself at high concentrations. The blockages produce a characteristic component with a mean of about 5 ms in the distribution of shut times (so k_{-B}≈1/5 ms=200 s^{-1}). Blockages become more frequent (the relative area of the 5 ms component increases) with concentration. *Closed circles*: reciprocal of the corrected mean open time plotted against concentration (slope 3.9×10^7 M^{-1} s^{-1}, intercept 594 s^{-1}). *Open circles*: the 'blockage frequency plot' - the frequency of blockages per unit of open time is plotted against concentration (slope=3.8×10^7 M^{-1} s^{-1} , intercept 4 s^{-1}). Reproduced with permission from Ogden & Colquhoun (1985).

of the blocker (it is still 1/α as it would be in the absence of the blocker). This prediction is also useful for testing the fit of the results to the postulated mechanism (e.g. Neher, 1983). The basis of this prediction is outlined by Colquhoun & Hawkes (1983); it is as though the opening 'carried on as normal' after a blockage. However this way of thinking of the blocking effect is clearly incompatible with a memoryless process; the channel cannot 'know' how long it has been open during earlier openings in the burst. And it can lead to errors; for example it might be supposed that if we separated out from the record all of those channel activations in which no blockage happened to occur then such activations would not be 'cut short' so they would have a normal mean open time of 1/α (the number of blockages per activation is random, there may be 0, 1, 2, . . . blockages; see Fig. 8). This is not true: in fact openings thus selected would have a shortened mean lifetime of 1/(α+$k_{+B}x_B$) just like the openings that were terminated by a blockage (see Colquhoun & Hawkes, 1983 for a fuller discussion).

The blockage frequency plot

When activations of the channel are frequent (at high agonist concentrations) the beginning and end of a burst will not be clearly distinguishable, so the prediction that the mean total open time per burst is unaltered by the channel blocker cannot be

tested. However a similar test can be achieved by doing a 'blockage frequency plot' (Ogden & Colquhoun, 1985), as long as a component that is attributable to blockages can be distinguished in the shut time distribution. By analogy with (2.5), the frequency of channel blockages should be $p_1 k_{+B} x_B$ where p_1 is the fraction of open channels. Thus, if the frequency of blockages per unit open time is plotted against the blocker concentration the slope of line should be k_{+B}. An experimental example of such a plot is shown in Fig. 7 (open circles); it is seen to behave as predicted by the simple channel block mechanism. The slope of this line gives $k_{+B}=3.8\times10^7$ M^{-1} s^{-1}, a very similar value to that estimated from the shortening of openings.

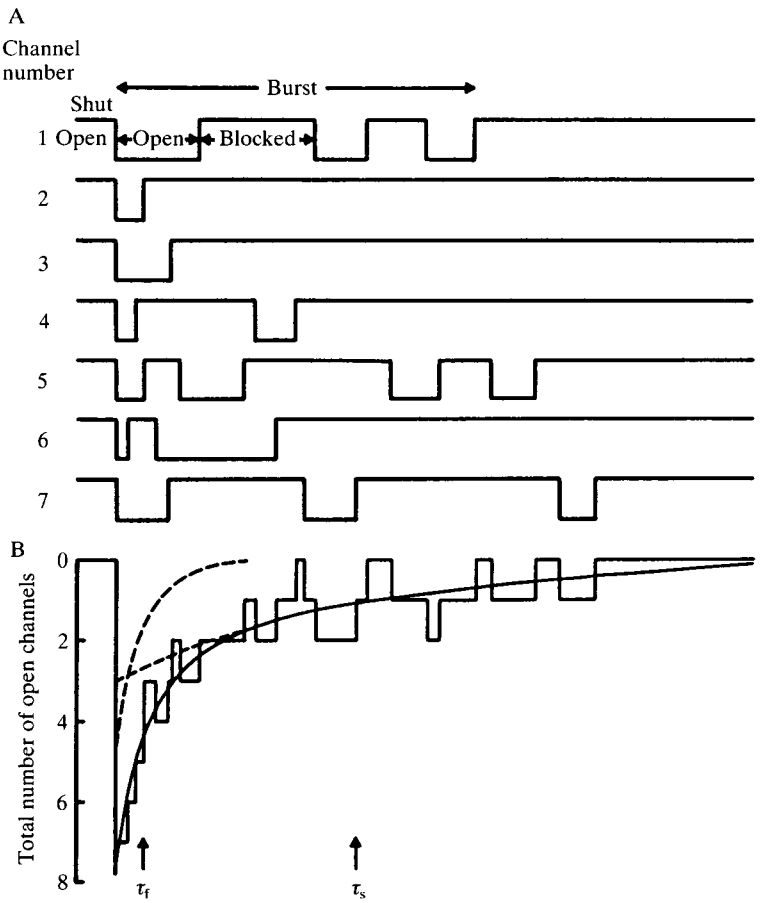


Fig. 8. Simulation of MEPC decay as in Fig. 3, but in this case a channel blocker is supposed to be present so some of the openings (all but channels 2 and 3) are interrupted by one or more blockages. The sum of all seven channels is shown in the lower part; a double exponential decay curve is superimposed on it (the two separate exponential components are shown as dashed lines).

Relationship between bursts and macroscopic currents with channel block

The relationship between bursts at the single channel level, and double exponential relaxation at the macroscopic level is illustrated by the simulated MEPC in Fig. 8.

As in Fig. 3, the seven channels that are illustrated open almost synchronously, but this time the opening may be interrupted by blockages before the agonist dissociates and the channel shuts. For example, channel 1 has two such blockages, channel 5 has three blockages, and channels 2 and 3 have no blockages. When the channels are summed the total current is seen to decay with a double exponential time course (as illustrated in the experiment in Fig. 5). There is an initial rapid decay as channels shut, or are blocked for the first time, and then a much slower decay the rate of which reflects (approximately) the length of the whole burst of openings. The latter follows from (5.7), and the definition of b and c in (5.8) and (5.9). These give, if the agonist concentration is small enough (i.e. β' is small enough), and the mean burst length is long compared with the length of a blockage,

$$\tau_s = 1/\lambda_s \approx -b/c$$

$$\approx \frac{1}{k_{-B}} + \frac{1 + x_B/K_B}{\alpha} \tag{5.17}$$

The second term on the right hand side is the mean burst length, as will now be shown (see equation 5.23). Before doing this, it is necessary to consider the number of blockages that occur in a burst.

The number of blockages per burst. First note that the number of openings in a burst must always be one greater than the number of blockages, so we shall actually work with the number of openings per burst. This will, like everything else, be random. The distribution of the number of openings per burst, and hence an expression for the mean number, can be derived as follows. Consider an open channel (state 1 in scheme (4.4)). Its next transition may be *either* blocking (going to state 2 with rate $k_{+B}x_B$), or shutting (going to state 3 with rate α). The relative probability of the former happening (regardless of how long it takes before it happens), which we shall denote π_{12}, is thus

$$\pi_{12} = \frac{k_{+B}x_B}{\alpha + k_{+B}x_B} \tag{5.18}$$

and the probability of the latter happening is therefore $\pi_{13}=1-\pi_{12}$. Notice also that a blocked channel (state 2) *must* unblock (to state 1) eventually, so $\pi_{21}=1$. The probability of blocking and then reopening is therefore $\pi_{12}\pi_{21}=\pi_{12}$. A burst will contain r openings (and $r-1$ blockages) if an open channel blocks and unblocks $r-1$ times (probability $\pi_{12}{}^{r-1}$), and then returns after the last opening to the long- lived shut state, state 3 (with probability π_{13}). The probability of seeing r openings (i.e. $r-1$ blockages) in a burst is thus

$$P(r) = \pi_{12}^{r-1}\,\pi_{13}\,. \tag{5.19}$$

This form of distribution is known as a *geometric distribution*. It is the analogue, for a discontinuous variable, of the exponential distribution, and has the same shape as an exponential distribution except that it decays in steps, rather than continuously.

One or other of the possible outcomes (r=1, 2, 3, . . .,∞) must occur, so these probabilities must add to unity, i.e., from (5.19),

$$\sum_{r=1}^{\infty} P(r) = 1\,. \tag{5.20}$$

The mean number of openings per burst can be found from the general expression for the mean, μ, of a discontinuous distribution, *viz.*

$$\mu = \sum_{r=1}^{\infty} rP(r)\,. \tag{5.21}$$

In the present example, the mean number of openings per burst is therefore

$$\mu = \frac{1}{1-\pi_{12}} = 1 + \frac{k_{+B}x_B}{\alpha}\,. \tag{5.22}$$

As expected this depends simply on the relative probabilities of leaving the open state for the blocked, or the shut, states. The mean number of blockages per burst, $k_{+B}x_B/\alpha$, is directly proportional to the blocker concentration.

We can now work out the mean length of a burst. The average burst consists of μ openings, each of mean length m_1, and μ–1 blockages, each of mean length m_2 (as defined in (4.5)). The mean burst length is therefore

$$\mu m_1 + (\mu - 1)m_2 = \frac{1 + x_B/K_B}{\alpha}\,. \tag{5.23}$$

This should increase linearly (from 1/α) with the blocker concentration.

Bursts with agonists alone

One reason for expecting openings to occur in bursts has been discussed above, *viz.* multiple openings during a single occupancy. Whatever the reasons the phenomenon is certainly common, as illustrated in Fig. 6. A typical histogram of all shut times for an agonist activated channel is given, and discussed, in Chapter 6 (Fig. 10).

If we assume that a mechanism like (4.1) is what underlies these observations, then we can extract information from the results by two sorts of measurements. It has already been mentioned (4.2) that the mean length of the short gaps within a burst will be approximately $1/(\beta+k_{-1})$. This alone does not allow us to estimate the separate values of β and of k_{-1} . However it is also true (see below) that the mean number of

openings per burst should be $1+\beta/k_{-1}$. If this is measured too, then the channel opening rate constant β, and the agonist dissociation rate constant k_{-1} can both be separately estimated (e.g. Colquhoun & Sakmann, 1985).

The number of openings per burst for agonist alone. The distribution of the number of openings per burst can be derived in much the same way as in the case of bursts caused by channel blockages, in (5.18) - (5.22). Consider a channel in the intermediate state AR (state 2 in scheme (4.1)). Its next transition may be *either* reopening (rate β) or agonist dissociation (rate k_{-1}). The relative probability of the former happening (regardless of how long it takes before it happens), which we shall denote π_{21}, is thus

$$\pi_{21} = \frac{\beta}{\beta + k_{-1}} \tag{5.24}$$

and the probability of the latter happening is therefore $\pi_{23}=1-\pi_{21}$. A burst must contain at least one opening; for the channel to *re*-open $r-1$ times before dissociation occurs the former event must occur $r-1$ times before the latter happens. The probability of r openings in a burst (r=1, 2, 3, ..., ∞) is thus

$$P(r) = \pi_{21}^{r-1}\pi_{23} \tag{5.25}$$

This is the geometric distribution of the number of openings per burst. The mean number of openings per burst is thus, from (5.21)

$$\mu = 1/\pi_{23} = 1 + \beta/k_{-1}\,, \tag{5.26}$$

as asserted above.

The actual molecular transitions that underlie the bursting behaviour in scheme (4.1) are illustrated explicitly in Fig. 9. Notice the 'invisible' oscillations between the

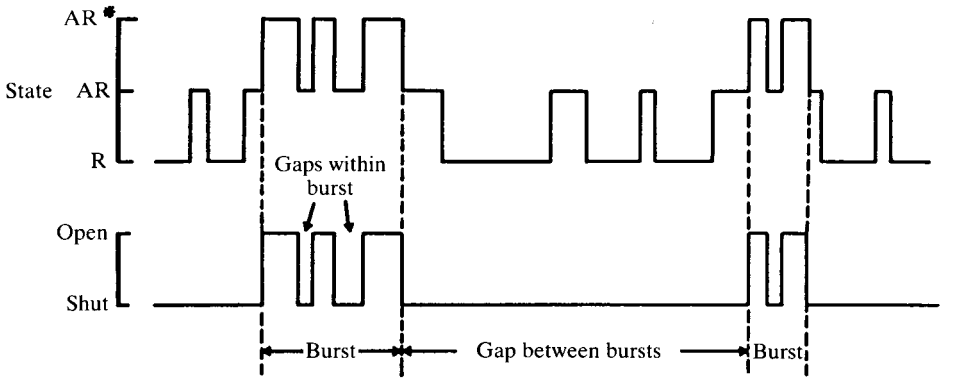


Fig. 9. Schematic illustrations of transitions between various states (top) and the corresponding observed single channel currents (bottom) to illustrate the origin of bursting behaviour for the simple agonist mechanism in (4.1). Notice that many transitions between shut states are invisible on the experimental record.

two shut times (mentioned above) that result from agonist occupancies that fail to produce opening of the channel. A more elaborate version of this diagram is given by Colquhoun, Ogden & Cachelin (1986) who use it to illustrate what happens when either the binding step is very fast (see above) or, alternatively, when the open-shut conformation change is very fast.

6. Correlations in single channel records

It was assumed above that the future evolution of a system depends only on its present state and not on its past history. For the simple open-shut model in (2.1) this implies, for example, that the length of an opening must be quite independent of (and therefore uncorrelated with) the length of the preceding shut time and of the lengths of preceding open times. The same is true of the 3-state mechanisms in (4.1) and (4.4); furthermore there will be no correlation between the length of one burst of openings and the next for either of these mechanisms. However more complex mechanisms may show such correlations; they are still 'memoryless', so sojourns in *individual* states will be independent, but correlations can arise because it is not possible to distinguish one sort of shut state from another on the record (they all have zero conductance), and similarly open states of equal conductance are not distinguishable. In particular, there will be correlations if there are (*a*) at least two open states (*b*) at least two shut states and (*c*) at least 'two routes' between the shut states and the open states (Fredkin, Montal & Rice, 1985; Colquhoun & Hawkes, 1987; Ball & Sansom, 1988).

Correlations between open times and between shut times

The last condition for the appearance of correlations, that there should be at least 'two routes' between the shut states and the open states, must now be put rather more precisely. Correlations will be found if there is no single state, deletion of which totally separates the open states from the shut states. The number of states which must be deleted to achieve such a separation is the *connectivity* of open and shut states, so correlations will be seen if the connectivity is greater than one. The mechanisms in (6.1) show three mechanisms each with

(6.1)

two open states (denoted O) and three shut states (denoted C). In schemes (*a*) and (*b*) there will be no correlations; deletion of state C_3 (or of state O_2) in (*a*) separates the open and shut states, as does deletion of C_3 in (*b*). In (*c*), on the other hand, the

connectivity is 2 (e.g. deletion of C_3 *and* C_4 will separate open and shut states) so correlations between open times may be seen. Even in this case correlations between successive open times will be seen only if the two open states, O_1 and O_2, have different mean lifetimes. The correlations result simply from the occurrence of several $C_4 \rightleftharpoons O_1$ oscillations followed by a $C_4 \rightarrow C_3$ transition and then several $C_3 \rightleftharpoons O_2$ oscillations, so runs of O_1 and runs of O_2 openings occur. The effect will clearly be most pronounced if the $C_4 \rightleftharpoons C_3$ reaction is relatively slow.

For example, most of the properties of the nicotinic receptor are predicted well by (*c*): in this case O_2 has a long mean lifetime compared with O_1 (but it has the same conductance), whereas C_3 has a very short lifetime. Thus long open times tend to occur in runs (so there is a positive correlation between the length of one opening and the next), but long openings tend to occur adjacent to short shuttings, giving a negative correlation between open time and subsequent shut time Colquhoun & Sakmann (1985).

In the earlier work in this field, it was usual to measure correlation coefficients from the experimental record. However it is visually more attractive, and in some respects more informative, to present the results as graphs, as suggested by McManus, Blatz & Magleby (1985), Blatz & Magleby (1989), and Magleby & Weiss (1990). An example of such a plot is shown in Fig. 10.

This graph illustrates correlations found for the NMDA-type glutamate receptor (Gibb & Colquhoun, 1992). To construct this graph, five contiguous shut time ranges were defined (each range being centred around the time constant of a component of the shut time distribution). Then, for each range, the average of the open times was calculated for all openings that were adjacent to shut times in this range, and this average open time was plotted against the mean of the shut times in the range. The

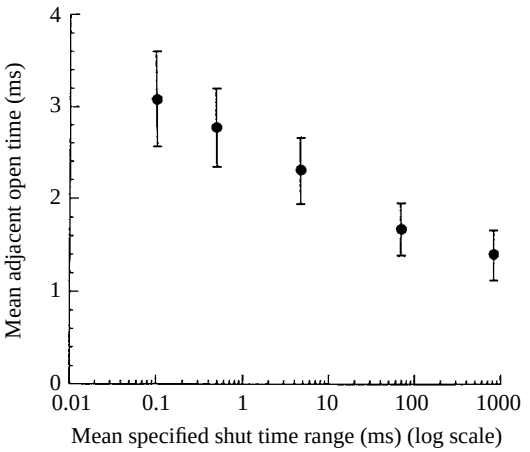


Fig. 10. Relationship between the mean durations of adjacent open and shut intervals. The graph shows the mean ± standard error of the open times in sixteen different patches, plotted against the average of the mean adjacent shut time ranges used in each patch. Reproduced from Gibb & Colquhoun (1992).

graph in Fig. 10 shows a continuous decline, so it is clear that long open times tend to be adjacent to short shut times, and *vice versa*.

In principle the connectivity between open and shut states can be measured experimentally because the correlation between an open time and the *n*th subsequent open time (for a single channel) will decay with increasing lag (*n*) towards zero as the sum of *m* geometric terms, where *m* is the connectivity minus one (Fredkin *et al.*, 1985; Colquhoun & Hawkes, 1987). However such a quantitative interpretation of correlations has not yet been achieved in practice.

These results can be extended to correlations between the lengths of bursts of openings, and between the lengths of openings within a burst (Colquhoun & Hawkes, 1987). There will be correlations between bursts when the connectivity (as defined above) between open states and *long-lived* shut states is greater than one. There will be correlations between openings within a burst when the *direct* connectivity between open states and *short-lived* shut states is greater than one (the term *direct connectivity* refers only to routes that connect open and short-lived shut states directly, not including routes that connect them indirectly *via* a long lived shut state, entry into which would signal the end of a burst). Thus, for the examples in (6.1), taking C_5 to be the long lived shut state, neither (*a*) nor (*b*) would show any such correlations, whereas (*c*) would show correlations within bursts but no correlations between bursts (as observed experimentally by Colquhoun & Sakmann, 1985). The following scheme (in which C_5 and C_6 both represent long lived shut states), on the other hand, would show all three types of correlation

(6.2)

7. Single channels after a voltage- or concentration-jump

The lifetime of a sojourn in a single state will always (under our assumptions) follow an exponential distribution. However some sorts of measurements may be dependent on whether the recording is made at equilibrium or not. So far we have supposed that recordings are made at equilibrium. Strictly speaking the assumption is a bit less strong - we assume that there is a steady state; however, for the sort of mechanism we are discussing this amounts to the same thing (Colquhoun & Hawkes, 1982, 1983).

Some new, and more complex, considerations arise when the record is not at equilibrium. For example, following a sudden change in concentration (a *concentration-jump*) or membrane potential (a *voltage-jump*), it will take some time for a new equilibrium to be established. It is usual to look at the channel openings (or shuttings, or bursts) that follow such a perturbation as though the 1st, 2nd, . . .

opening (or shutting, or burst) were all directly comparable. This will be the case if there is only one sort of shut state and one sort of open state as in the simplest scheme (2.1) that was discussed earlier (Colquhoun & Hawkes, 1987).

Consider, for example, the case where the channels are shut initially but may open following a depolarizing voltage step applied at t=0. If there is more than one sort of shut state the latency until the first opening may depend on how the system was distributed among the various shut states at t=0, so measurements of the first latency can give information about this, and about the lifetimes of these shut states. Thereafter, however, every open time and shut time will be exactly comparable, each having exactly the same distribution as it would have in an equilibrium record. This simple result will happen in precisely those cases in which open and shut times are not correlated (as described in the preceding section).

When open and shut times are correlated then the lengths of the 1st, 2nd, . . . etc. openings (or shuttings, or bursts) will not all have the same distribution, though they will approach the equilibrium distribution eventually. Their distributions should, however, all have the same observed time constants; these time constants are functions (though possibly quite complicated functions) *only* of the underlying transition rates and, according to our assumptions, these remain constant throughout the post-jump period, because the membrane potential and/or ligand concentrations are supposed to remain constant. What is happening is that the occupancies of each state, and hence, from (2.5) and (2.6), the actual transition frequencies, are varying with time. This means that the relative *areas* of the components associated with each time constant may be different for the 1st, 2nd, . . . *etc*. open time following the jump. This phenomenon is discussed and exemplified by Colquhoun & Hawkes (1987), but it has not yet been exploited experimentally.

It is worth noting that voltage jumps and concentration jumps are usually quite brief (often in the range 1-300 ms), so they are not a good way of investigating slow kinetic processes (such as the so-called mode changes), which are necessarily obscured in short recordings.

The time course of synaptic currents

In many (though possibly not all) cases it seems that the time course of the post-synaptic current elicited by nerve stimulation is quite long compared with the length of time for which the transmitter is present in the synaptic cleft (e.g. Anderson & Stevens, 1973). In other words the presynaptic ending provides a very brief concentration jump of transmitter; this opens some channels which subsequently shut in the absence of transmitter. In the simulations in Figs 3 and 8 it was supposed that channels open essentially synchronously, i.e. that the latency to the first opening (*first latency*, for short) following exposure to the agonist is very short. This is probably close to the truth for fast channels such as the muscle-type nicotinic acetylcholine receptor (e.g. Franke *et al*., 1991; Liu & Dilger, 1991), and may well be true for at least some AMPA-type glutamate receptors in the CNS (Colquhoun, Jonas & Sakmann, 1992).

However it is clearly *not* true that channels open synchronously for the NMDA-

type glutamate receptor (Edmonds & Colquhoun, 1992); in this case the first opening may occur hundreds of milliseconds after a brief pulse of glutamate is applied. The consequences of this for the time course of the synaptic curent can be illustrated by the following oversimplified example.

Consider a hypothetical channel which, after brief agonist application, produces an activation consisting of a single opening, after the first latency has elapsed (for the NMDA receptor, the activation is actually a great deal more complicated than a single opening). In Figure 11A, nine examples are shown of simulated channels with a mean first latency of 1 ms, and a mean open time of 10 ms (the variability of both being described by simple exponential distributions).

The average current (shown at the top), is seen, not surprisingly, to have a rising phase that can be fitted with an exponential with a time constant of about 1 ms, and the decay phase has a time constant of about 10 ms. Apart from being about 10 times too slow, this example is similar to what happens at a neuromuscular junction.

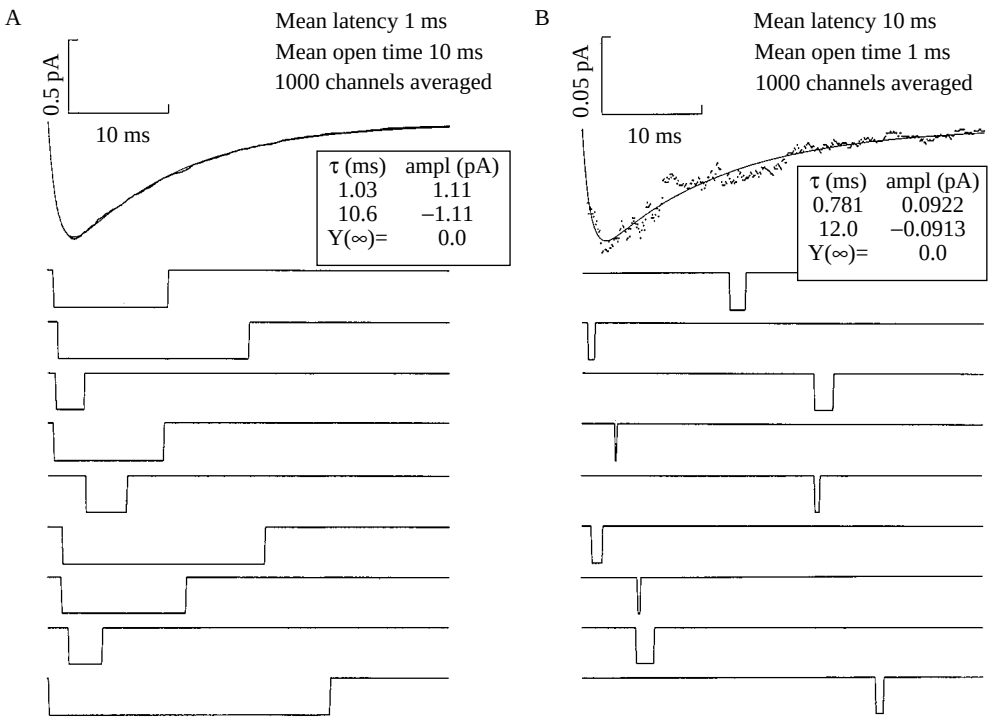


Fig. 11. A simple simulation of a synaptic current, in which each channel is supposed to produce a single opening only, after an exponentially-distributed latency. (A) Mean latency 1 ms, mean open time 10 ms. The lower part shows nine examples of simulated channels. The top trace is the average of 1000 such channels; the double-exponential curve fitted to the average has τ=1.03 ms (amplitude 1.11 pA), and τ=10.6 ms (amplitude=–1.11 pA). (B) Similar but with a mean latency of 10 ms and a mean open time of 1 ms. The double-exponential curve fitted to the average has τ=0.76 ms (amplitude 0.092 pA), and τ=12.0 ms (amplitude=–0.091 pA).

More surprising, perhaps, are the results shown in Figure 11B, in which the numbers are reversed, and simulated channels have a mean first latency of 10 ms, and a mean open time of 1 ms. The averaged current shown at the top is seen to have essentially the same shape as in Figure 11A (though it is ten times smaller, and considerably noisier relative to its amplitude). Thus, in this case, *the rate of decay reflects the duration of the first latency, whereas the rate of rise represents the mean channel open time* (this case resembles observations on sodium channels: Aldrich, Corey & Stevens, 1983). The reason for this result, which seems paradoxical at first sight, can be seen from the simulations (e.g. the exponential distribution of first latencies means that short latencies are more common than long ones), and from the relevant theory which was outlined above. The distribution of the time from the stimulus until the channel shuts finally is simply the distribution of the sum of (*a*) the first latency (mean length $1/k_{21}$ say), and (*b*) the length of the channel opening (mean length $1/k_{12}$ say). Since both have been taken to be simple exponentials, as defined in (3.12) and (3.11) respectively, this distribution is given by the convolution in (3.13). The result, $f(t)$, has already been given in (3.14), and illustrated in Fig. 4. It has the form of the difference between two exponentials, which is what has been fitted to the averages in Fig. 11.

In this particular simple case, though not in general, there is a very simple relationship between the distribution, $f(t)$, of the total event length, and the shape of the averaged current. The time course of the current is given, apart from a scale factor, by the probability that a channel is open at time t. Now a channel will be open at time t if (*a*) the first latency is of length u, *and* (*b*) the channel stays open for a time *equal to or greater than* $t-u$. The probability that a channel stays open for a time $t-u$ *or longer*, is, from (3.11), the cumulative distribution

$$F_1(t-u) \equiv e^{-k_{12}(t-u)} \tag{7.1}$$

(see Chapter 6, equations 6.4-6.5), so, by an argument exactly like that used to arrive at (3.13), the probability that a channel is open at time t is

$$P_{\text{open}}(t) = \int_{u=0}^{u=t} f_2(u)F_1(t-u)\,\mathrm{d}u \tag{7.2}$$

This differs from (3.14) only by a factor of $1/k_{12}$=mean open lifetime, so

$$P_{\text{open}}(t) = f(t)/k_{12} \tag{7.3}$$

which is, apart from its amplitude, unchanged when k_{12} and k_{21} are interchanged The amplitudes of the two exponential components are equal and opposite, being, from (3.14),

$$a = k_{21}/(k_{21}-k_{12})\,, \tag{7.4}$$

with a maximum at $t_{\max}$ defined in (3.15). The simulated average currents in Fig. 11 are indeed well-fitted by these values.

It is clear from this discussion that observations on the average (macroscopic)

current alone cannot tell us whether the slow decay of an observed average results from a long first latency, or from a long channel opening (see, for example, Edmonds & Colquhoun, 1992, for experimental results). In order to distinguish between these possibilities it is necessary to measure either the first latency distribution, or the length of channel activations (or preferably both) independently. In practice this may not be as easy as it sounds (*a*) because the channel activations are usually more complicated than the single openings assumed here (in the case of the NMDA type of glutamate receptor they are *much* more complicated), and (*b*) because the measurements must be made on a patch of membrane that contains only one ion channel molecule (or at least a known number of molecules), and this is not easy to achieve in practice.

8. The problems of missed events and inference of mechanisms

Inferring reaction mechanisms

The discussion so far has concentrated on looking at the behaviour of specified reaction schemes. However the most important question of all comes at an earlier stage, namely, what is the qualitative nature of the reaction mechanism? The first stage is usually to inspect the distributions of open times and shut times. If these distributions can be fitted well with a sum of exponential components then the number of components required can be taken as a (minimum) estimate of the number of open states and the number of shut states, respectively. If the record can clearly be divided into bursts of openings then other distributions can be investigated, for example the distribution of the burst length, the distribution of the number of openings per burst, and the distribution of the total open time per burst. The last two should both have a number of components equal to the number of open states, and the distribution of the total open time per burst is of particular interest because, unlike the distribution of open time, it is relatively little affected by inability to detect brief shuttings of the channel.

The next question concerns how the open and shut states are connected to each other. The measurement of correlations between openings, bursts etc. may cast light on this problem, as may investigation of the distributions at various times after a voltage jump or other perturbation. In addition the presence or absence of a component of isolated openings in the distribution of the number of openings per burst may be informative (Colquhoun & Hawkes, 1987). However the single channel record does not, in principle, contain sufficient information to allow all of the connections to be worked out unambiguously. Single channel analysis, just like all other experimental work (whether biochemical or physiological) procedes by erecting hypotheses and then trying to demolish them with experimental results that are incompatible with the predictions of the hypotheses.

Missed-event corrections based on a postulated mechanism

The problems that arise from the inability to detect, in practice, the briefest openings and shuttings has already been discussed, from the practical point of view, in Chapter 6 (§10). It was pointed out there that if substantial numbers of both openings and shuttings were missed, then it was possible to correct for this imperfection only in cases where a kinetic mechanism could be postulated for the channel. Even then there may be no unique solution of the problem (Colquhoun & Sigworth, 1983). Corrections based on a postulated mechanism will be discussed next.

When openings and shuttings are missed in substantial numbers, the distributions of the *observed* (inaccurate) open and shut times are no longer expected to be described by a mixture of exponentials, as has been assumed throughout, and the theory gets a good deal more complicated. Several approximate methods for dealing with the problem have been suggested, e.g. Roux & Sauvé (1985), Wilson & Brown (1985), Blatz & Magleby (1986), Ball & Sansom (1988), Milne *et al.* (1989) and Crouzy & Sigworth (1990). However an exact solution to the problem (as usually formulated) was found by Hawkes, Jalali & Colquhoun (1990) (their calculations suggest that the best of the approximations is that of Crouzy & Sigworth). Calculation of the exact result for short times (where it is simple), combined with use of an asymptotic approximation (Hawkes, Jalali & Colquhoun, 1992) at longer times, allows accurate prediction of the *observed* distributions for any specified mechanism and time resolution. Such calculated distributions will be referred to as HJC distributions.

Fitting a mechanism directly to data

The ability to calculate the distribution of the quantities that are actually observed provides a way to correct for missed events. But in addition, it also opens the way to fitting a specified mechanism directly to the data. Previously all one could do (as in the examples in Chapter 6) was to fit empirical mixtures of exponentials separately to open times, shut times, burst lengths etc., which yielded values for *observed* time constants and areas; the corrections for missed events, the interpretation of the results in terms of a mechanism, and the estimation of the underlying 'mass action' rate constants (see §2) for the mechanism, all had to be done retrospectively. Now that missed events can be allowed for, it is possible to fit the HJC distributions directly to the *measured* open and shut times, with the adjustable parameters in the fit *not* being empirical time constants and areas, but the underlying rate constants for the model. Since the time resolution must be specified in order to do this, it becomes particularly important to ensure that this is known and consistent, as discussed in Chapter 6 (§5).

Simultaneous maximum likelihood fitting

In fact it may be possible to do much better than this. It is a problem with the conventional approach to inference of mechanisms that there are many different sources of information to be collated. For example one may have distributions of open times, shut times, burst lengths, numbers of openings per burst, correlations, and many others. Furthermore, the information from some sorts of fit overlaps heavily

(e.g. the distribution of total open time per burst may be very similar to the distribution of burst length) so it can be a problem to decide how many such things to calculate.

However, once one can calculate the probability (density) for the *observed* open and shut time, by means of the HJC distributions, or approximations to them, then it becomes feasible to calculate the *likelihood* (see Chapter 6) of an entire single channel record (see Horn & Lange, 1983; Horn & Vandenberg, 1984; Fredkin & Rice, 1992). This means that, in order to do the fitting, we do not have to fit separately all of the sorts of distribution mentioned above; instead we simply adjust the parameters (mass action rate constants) so as to maximise the likelihood for the entire record. This takes into account *simultaneously* the information from the open times, from the shut times and from *the order in which they occur* (i.e. from the burst structure and from correlations between events). We have found this to be quite feasible on a fast PC, at least for a few thousands of observations. Further than this, it also becomes feasible to fit several different sorts of measurement simultaneously, for example recordings made with different concentrations of agonist (Hawkes *et al.*, in preparation).

This approach can be used to judge the relative merits of alternative postulated mechanisms. If each of the proposed mechanisms is fitted to the same data, the relative plausibility of each mechanism can be assessed from how large its likelihood is. This has been done, for example, by Horn & Vandenberg (1984) (without missed event correction).

An example of fitting with missed events

An example of maximum likelihood fitting by the HJC method (to simulated single channel data) is shown in Fig. 12.

The data in the histograms in Fig. 12 were simulated using the 5-state model for the nicotinic acetylcholine receptor, with the rate constants and concentration used by Colquhoun & Hawkes (1982) as shown in (8.1). The numbers are transition rates in s^{-1} (see discussion following 2.1).

(8.1)

A resolution of 100 μs (for both open and shut times) was then imposed on the

simulated sequence of open and shut times (see Chapter 6), to produce a sequence of *apparent* open and shut times from which the histograms were constructed. These histograms were not themselves fitted, but the likelihood of the entire sequence of *apparent* open and shut times was calculated, as outlined above, and the free

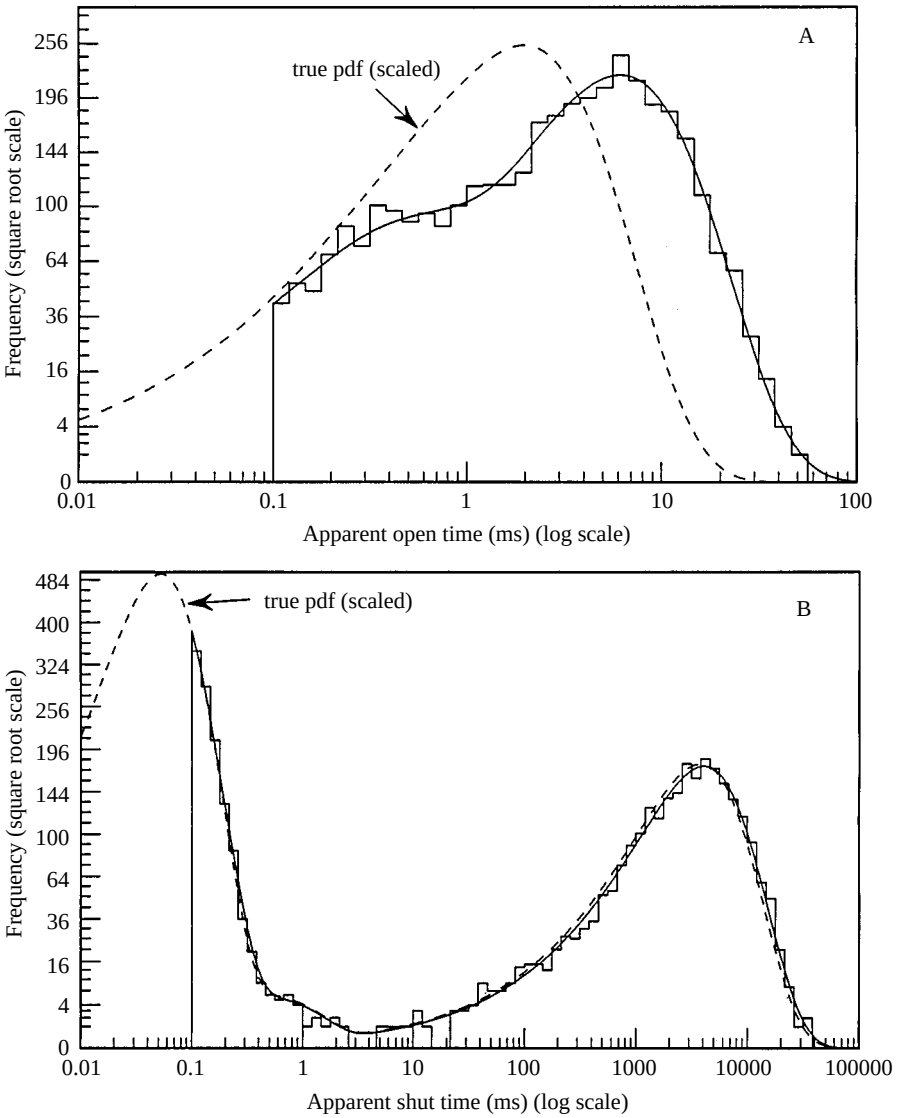


Fig. 12. Example of the effect of missed events. The 5-state mechanism in (8.1) was used to simulate 10235 channel transitions. A resolution of 100 μs was imposed on the simulated channels resulting in 3419 resolved intervals, from which the histograms of apparent open tines (A), and apparent shut times (B) were constructed. The continuous lines are the HJC distributions calculated for a resolution of 100 μs with rate constants as in (8.1); the exact solution is used for intervals up to 300 μs, and the asymptotic solution thereafter. The dashed lines show the true open and shut time distributions.

parameters adjusted (by the simplex method) to maximize this likelihood. The estimates of the transition rates, from this *single* fitting procedure, were close to the values shown in (8.1), as hoped. The true values from (8.1) were then used to calculate the HJC distributions for the *apparent* open times and shut times. These are shown as continuous lines superimposed on the histograms in Fig. 12. It can be seen that they fit the 'observations' quite well. The same values were then used to calculate the predicted open and shut time distributions by the standard methods (e.g. Colquhoun & Hawkes, 1982), with no allowance for missed events. These distributions, which are what would be expected if we had perfect time resolution, are shown as dashed lines in Fig. 12A,B.

The open time distribution in Fig. 12A shows that the openings appear to be considerably longer than their true values, because many brief *shuttings* are below the 100 μs resolution, and therefore not detected. The true fast shut time component has τ=52.6 μs, so 85% of this component is missed. With perfect resolution the open time distribution would, with the rate constants in (8.1), have two exponential components with τ=2.0 ms (93% of area) and τ=0.33 ms (7% of area). The asymptotic HJC distribution of apparent open times, on the other hand, has components with τ=6.1 ms (81% of area) and 0.33 ms (19% of area).

The shut time distribution in Fig. 12B shows, in contrast, that the distribution of apparent shut times is close to the true distribution, except of course that there are no shut times below 100 μs: this happens because, in this particular example, relatively few *openings* are below 100 μs. This shows, incidentally, that for results of this sort, the number of missed shuttings can be estimated quite accurately by extrapolating the shut time distribution to zero length. Thus the crude form of retrospective missed event correction used by Colquhoun & Sakmann (1985) (see also Chapter 6) should have been reasonably accurate.

Problems: the number of channels

Perhaps the biggest single problem that hinders the interpretation of single channel records stems from the fact that one rarely knows how many functional channels were present in the membrane patch from which the record was made. Shut time distributions, including first latency distributions, must obviously depend on the number of channels that are present.

If it is observed, at any time during the recording, that more than one channel is open, then the patch must contain more than one channel, but it is notoriously difficult to estimate how many there are, at least when P_{open} is low (see Horn, 1991, and Chapter 6, §5). A method based on the length of runs of single openings has been proposed (Colquhoun & Hawkes, 1990), which may be useful if the burst structure of the data is not too complex.

Perhaps the most common way of managing this problem is to confine attention only to subsections of the record that *can*, with confidence, be attributed to the activity of only one channel. Such subsections may consist of individual bursts (e.g. at low agonist concentrations), or may consist of much longer clusters of openings when

P_{open} is high (see Chapter 6). This means, of course, that information from longer shut periods is lost. This approach can be incorporated into maximum likelihood fitting with HJC distributions (Hawkes, Jalali, & Colquhoun, in preparation).

Problems: indeterminacy of parameters

It is unlikely that any individual experimental record will contain enough information to allow estimation of *all* the rate constants in the postulated mechanism. This problem can be minimized if several sorts of experiment (e.g. equilibrium records with different ligand concentrations, jump experiments, P_{open} curves etc.) can be analysed simultaneously, as described above. But in practice it will often be necessary to estimate some rate constants form one sort of experiment, and then to treat these values as fixed while estimating others from a different sort of experiment (see, for example, Colquhoun & Sakmann, 1985; Sine, Claudio & Sigworth, 1990).

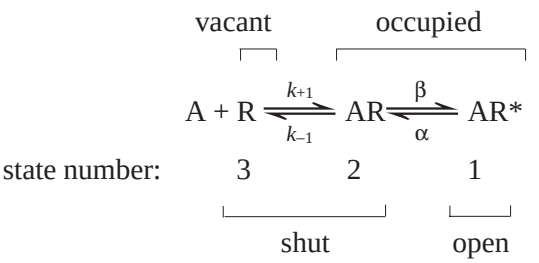
Problems: indeterminacy of mechanisms

It is well-known that kinetic mechanisms are not unique. Whatever mechanism is proposed, it will virtually always be possible to find another mechanism that fits the experimental results just as well. Some classes of mechanism predict results that are too similar for them to be distinguished in practice. Worse still, some classes of mechanism are indistinguishable in principle; this has been discussed very elegantly by Kienker (1989). Nevertheless, combination of different sorts of biophysical experiment (as in the last paragraph), together with biochemical and structural information, can do much to reduce the ambiguity.

These problems lead some people to be pessimistic about the possibility of interpreting experiments in terms of physical mechanisms. One extreme reaction is to point out that proteins have an essentially infinite number of conformations (which is, no doubt, true), and must therefore be analysed by fractal methods, with consequent denial that anything can be gleaned about the physical mechanisms involved (see. for example, Korn & Horn, 1988). We think that it is perverse to suggest that experimental results have not been able to cast light on topics such as mechanisms of ion channel block, or the fine structure of bursts; the proposed mechanisms are doubtless approximations, but they are not baseless.

Appendix 1. Derivation of the shut time distribution for the Castillo-Katz mechanism

The Castillo-Katz mechanism defined in (4.1) will be written again here, for easy reference.



(A1.1)

The derivation is fairly lengthy, even for this mechanism with only three states. This complexity results, to a great extent, from the fact that the two shut states can interconvert directly (unlike the simple channel block mechanism), so that there may be any number of oscillations between them during any individual shut period. It can easily be imagined that derivations such as that to follow are not generally feasible for mechanisms with more than three states, and even with three states the complexity is great enough to provide a considerable incentive to program the quite general matrix results (Colquhoun & Hawkes, 1982) which will give numerical results for *any* mechanism.

First, notice that when the channel is at equilibrium, *every* shut period follows an opening, and so must start with a sojourn in the intermediate complex, AR (state 2). After leaving AR the channel may reopen immediately, or it may go through any number of AR⇌R oscillations before reopening. Furthermore every shut period must end in state 2 (AR) also, because the shut period ends when an opening occurs, and opening is possible only from state 2.

We start by defining the probability, denoted $P_{22}(t)$, that the channel starts in state 2, stays shut (state 2 or 3) *throughout* the time period from 0 to t, and is in state 2 at t. The shut lifetime will be t if, having stayed shut throughout the time from 0 to t, the channel then opens. The probability that a channel opens during a small time interval, Δt, is, by analogy with (2.10), $\beta\Delta t$. The probability density function for all shut times, $f_s(t)$ say, can be found by multiplying these two probabilities (the probabilities are independent, because the probability of a transition from state 2 to state 1 in the interval between t and $t+\Delta t$ is independent of the previous history of the process): it is, therefore, the limiting value, for very small Δt, of the following expression.

$$\begin{aligned} f_s(t) &= \text{Prob[channel shut from 0 to } t \textit{ and} \text{ opens during } t,\, t+\Delta t]/\Delta t \\ &= P_{22(t)}\beta \end{aligned} \qquad \text{(A1.2)}$$

We can now get an expression for $P_{22}(t)$, in the following way. The channel will be in state 2 (AR) at time $t+\Delta t$ *if either* (1) it was in state 2 at time t, *and* fails to leave state 2 during Δt, *or* (2) it was in state 3 (R) at time t, *and* moves from 2 to 3 (i.e. binds a ligand molecule) during Δt. To evaluate the first of these contingencies, we note that the probability of leaving state 2 (for either state 3 *or* state 1) during Δt is $(\beta+k_{-1})\Delta t$, so the probability of *not* leaving is 1 minus this quantity. To evaluate the second term

we must first define $P_{23}(t)$ as the probability that a channel that is in state 2 at t=0 then remains shut throughout the time from 0 to t and is in state 3 at t; the probability of a transition from 3 to 2 in Δt is then $k_{+1}x_A\Delta t$. We can now assemble all the possibilities to write

$$P_{22}(t + \Delta t) = P_{22}(t)[1 - (\beta + k_{-1})\Delta t] + P_{23}(t)k_{+1}x_A\Delta t \, . \qquad \text{(A1.3)}$$

Thus

$$\frac{P_{22}(t+\Delta t) - P_{22}(t)}{\Delta t} = - P_{22}(t)(\beta + k_{-1}) + P_{23}(t)k_{+1}x_A \qquad \text{(A1.4)}$$

and so, letting $\Delta t \rightarrow 0$, we obtain

$$\frac{dP_{22}(t)}{dt} = - P_{22}(t)(\beta + k_{-1}) + P_{23}(t)k_{+1}x_A \qquad \text{(A1.5)}$$

This equation contains two unknowns, $P_{22}(t)$ and $P_{23}(t)$. To solve this problem we must go through an exactly analogous argument to obtain a second equation, viz.

$$\frac{dP_{23}(t)}{dt} = P_{22}(t)\, k_{-1} - P_{23}(t)k_{+1}x_A \, . \qquad \text{(A1.6)}$$

These two equations allow the two unknowns to be evaluated. To achieve this we eliminate $P_{23}(t)$ from the equations by using (A1.5) to obtain an expression for $P_{23}(t)$, which is then substituted into (A1.6). The result is a second order equation that involves only $P_{22}(t)$, thus

$$\frac{d^2P_{22}(t)}{dt^2} + (\beta + k_{-1} + k_{+1}x_A)\frac{dP_{22}(t)}{dt} + k_{+1}x_A\beta P_{22}(t) = 0 \qquad \text{(A1.7)}$$

If we define the (constant) coefficients, b and c, as

$$b = \lambda_1 + \lambda_2 = \beta + k_{-1} + k_{+1}x_A \qquad \text{(A1.8)}$$

$$c = \lambda_1\lambda_2 = k_{+1}x_A\beta \qquad \text{(A1.9)}$$

then (A1.7) can be written in the form

$$\frac{d^2P_{22}(t)}{dt^2} + b\frac{dP_{22}(t)}{dt} + cP_{22}(t) = 0 \qquad \text{(A1.10)}$$

We are now in a position to obtain expressions for the observed time constants. A solution of (A1.10) is needed, and we proceed, in the irritating manner of mathematicians, to propose, for no apparent reason, that there is a particular solution of (A1.10) that has the form $P_{22}(t)=e^{-\lambda t}$. If this is the case then $dP_{22}(t)/dt=-\lambda e^{-\lambda t}$, and $d^2P_{22}(t)/dt^2=\lambda^2 e^{-\lambda t}$. Substitution of these into (A1.10) gives:

$$\lambda^2 e^{-\lambda t} - b\lambda e^{-\lambda t} + c e^{-\lambda t} = 0 \qquad \text{(A1.11)}$$

This result must be true at all times, including at t=0. Putting t=0 in (A1.11) gives

$$\lambda^2 - b\lambda + c = 0 \qquad \text{(A1.12)}$$

This is a quadratic equation and therefore there will generally be *two* values of the *observed* rate constants, λ_1 and λ_2 say, that satisfy it, and these can be found as the two solutions of (A1.12), namely

$$\lambda_1, \lambda_2 = 0.5\left(b \pm \sqrt{b^2 - 4c}\right) \qquad \text{(A1.13)}$$

or

$$\lambda_1, \lambda_2 = \frac{2c}{b \mp \sqrt{b^2 - 4c}}, \qquad \text{(A1.14)}$$

where the coefficients, b and c, were defined in (A1.8) and (A1.9). Notice that these coefficients are rather simpler than those given in (5.4) and (5.5) for the macroscopic relaxation, because we are now dealing only with the shut states. They no longer involve *all* of the fundamental rate constants (α does not appear).

To complete the distribution of shut times, we must next find the *areas*, a_1 and a_2, of the two components. The p.d.f. has the form (see Chapter 6)

$$f_s(t) = \beta P_{22}(t) = a_1\lambda_1 e^{-\lambda_1 t} + a_2\lambda_2 e^{-\lambda_2 t} \qquad \text{(A1.15)}$$

where a_2=1–a_1, because the total are must be 1. Thus, from (A1.2) and (A1.15),

$$\left.\frac{df_s(t)}{dt}\right|_{t=0} = \left.\frac{\beta dP_{22}(t)}{dt}\right|_{t=0} = -a_1\lambda_1^2 - a_2\lambda_2^2$$

$$= a_1(\lambda_2^2 - \lambda_1^2) - \lambda_2^2$$

$$= -\beta(\beta + k_{-1}) \qquad \text{(A1.16)}$$

The last line follows from (A1.5) because $P_{22}(0)$=1 (state 2 cannot be left in zero time), and $P_{23}(0)$=0 (cannot get from state 2 to 3 in zero time). From this, together with the fact that $\lambda_1+\lambda_2=\beta+k_{-1}+k_{+1}x_A$ and $\lambda_1\lambda_2=k_{+1}x_A\beta$ (see A1.8 and A1.9), we find, after some manipulation, that the areas of the two components are given by

$$a_1 = \frac{\beta(k_{+1}x_A - \lambda_1)}{(\lambda_2 - \lambda_1)\lambda_1}, \quad \text{and} \quad a_2 = 1 - a_1 . \qquad \text{(A1.17)}$$

This completes the derivation of the distribution of all shut times. The same result was derived, in a quite different way, by Colquhoun & Hawkes (1981), but if all that is needed is numerical values for the time constants and areas of the components it is obviously much easier to use the entirely general result (Colquhoun & Hawkes,

1982), rather than go through a derivation like that above for every mechanism of interest.

Approximations for the time constants. If $b^2 \gg 4c$ then it follows from (A1.13) that one of the observed rate constants will be much larger than the other, say $\lambda_2 \gg \lambda_1$. This will be the case, for example, when the ligand concentration, x_A, is very low, so from (A1.8), we find that the faster time constant is approximately

$$\tau_2 = 1/\lambda_2 \approx \frac{1}{\beta + k_{-1}} \qquad \text{(A1.18)}$$

The right hand side of this is simply the mean lifetime of a single sojourn in the intermediate complex AR (state 2). This is, therefore, an example of a case where, contrary to the general rule, a physical interpretation *can* be placed on an observed time constant, as an approximation. In this case the sojourns in AR that occur as the channel oscillates between AR⇌AR*, can be seen (approximately) as the fast component of the distribution of all shut times.

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