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How fast do drugs work?

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Being a very-simplest introduction to those beautiful methods of reckoning which are generally called by the terrifying names of fluctuation and relaxation analysis.
(With acknowledgement to Silvanus P. Thompson, 1910)

It is my purpose to discuss some recently developed methods for the investigation of the rate of drug action; in particular, to discuss how the methods work, and what they tell us, rather than to review the many results that have been obtained by their use.

The nature of the problem

The question of how rapidly drugs interact with receptors was probably the first quantitative pharmacological question ever to be asked. While A. V. Hill was still a scholar of Trinity College Cambridge, he published, in 1909, a remarkable paper[8]. In this paper he obtained, 9 years before Langmuir, Langmuir's well-known result for the binding of a drug (nicotine and 'curari' in his experiments) to adsorption sites (the 'receptive substance' of muscle). He derived the relationship between the amount of binding at equilibrium and the free drug concentration; he also found the rate at which this equilibrium binding should be approached when drug was added to, or removed from, the tissue bath.

When first approaching the kinetics business, there are a number of obstacles to be overcome. One obstacle is the frightful jargon that has grown up around some aspects of the subject; another lies in the fact that this jargon is not always used accurately. A particularly common, and misleading, mistake is the confusion of the terms *kinetic, steady-state* and *equilibrium*. The proper definition of these ideas (in terms of entropy production) is not very helpful to most people, but their meanings can be illustrated by an analogy. Take, for example, the simple Michaelis-Menten mechanism for combination of enzyme (E) with substrate (S) to produce product (P), i.e. $E + S \rightleftharpoons ES \rightarrow P$. At equilibrium all the substrate will be used up; it will be completely converted to product (because the last reaction is irreversible). More generally, at *equilibrium* the forward and backward rates for each reaction step will be equal, and the amount of each reactant will be constant (not changing with time). A *steady-state* is defined by the second condition alone; for example it is well known that enzyme reactions will often, after a short transient period, reach an approximately steady state in which the concentration of enzyme–substrate complex varies little with time, even though the rate of association of substrate with enzyme, and dissociation of substrate from enzyme may be proceeding at quite different rates. The study of the *rates* of reactions, e.g. the rate at which equilibrium is approached is called *kinetics**. The reason that one often sees equilibrium studies referred to as 'kinetics' lies presumably in the fact that the equation which describes a simple enzyme reaction in the (quasi-) steady state bears a formal resemblance to the Hill–Langmuir equation for adsorption at equilibrium.

Another common bit of jargon is the term *relaxation*. When, for example, the drug concentration is suddenly changed, it is obvious that the occupancy of receptors will change, more or less gradually, until the equilibrium occupancy appropriate to the new drug concentration is attained. Instead of referring to this period of changing occupancy simply as re-equilibration it is often referred to as '*relaxation* of the system (towards equilibrium)'. And the sudden change of concentration is referred to as a concentration *jump*.

Simple binding reactions

Hill considered the simple binding reaction

$$A + R \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} AR \qquad (1)$$

in which A represents the drug, and R the receptor. The tendency of an unbound molecule to become bound is measured by the association rate constant, k_{+1}, which is defined by the law of mass action. When the drug concentration is x_A the effective association rate constant will be $k_{+1}x_A$. The tendency of the drug-receptor complex to dissociate is likewise measured by the dissociation rate constant, k_{-1}. The ratio of these rate constants ($k_{-1}/k_{+1} = K$) is the equilibrium constant, which is the concentration of drug at which half the binding sites are occupied at equilibrium†. One aim of a kinetic study would be to determine separately the two rate constants, k_{+1} and k_{-1}. Hill showed that when drug is added, the fraction of occupied sites (the *occupancy*) should approach its eventual equilibrium value along a simple exponential time course, and that the time constant (τ say) for this exponential curve (i.e. the half-time/0.693) should depend simply on the sum of the rate constants for association and dissociation, thus

$$\tau = 1/(k_{+1}x_A + k_{-1}) \qquad (2)$$

More generally, imagine that the drug concentration is suddenly changed from any original value, to some new value, x_A (this is what might, according to current fashion, be called a concentration jump experiment). The new concentration will correspond to a new equilibrium occupancy, and the transition to this new occupancy should follow an exponential time course with the time constant given above. Notice that the time constant gets shorter (i.e. re-equilibration gets faster) when the drug concentration is high, and that it becomes simply $1/k_{-1}$ when the drug concentration is zero, e.g. the drug is removed and the dissociation of bound drug is followed. Thus, observation of the re-equilibration rate, with a series of different drug concentrations, should allow the determination of the two rate constants, k_{-1} and k_{+1}, in the reaction mechanism.

Behaviour of individual receptors

It is easy to show in a formal mathematical way why re-equilibration should follow an exponential time course, but it is also possible, and much more illuminating, to explain this phenomenon in terms of the way that individual drug–receptor complexes behave. Each drug–receptor complex will not exist for the same fixed length of time; the lifetime of the complex will be *random*. The bonds holding the complex together will be stretching and bending very rapidly (on a picosecond timescale) as

* The term *pharmacokinetics* has, curiously, been sequestered by common usage to describe one small aspect of the subject – the disposition of drugs in the body. This hardly ever reflects the fundamental rate of drug action on receptors and therefore tells us nothing about *how* drugs work, though, as an empirical subject, it is useful in practice.

† Hill showed that the occupancy at equilibrium should be $x_A/(x_A + K)$, a result usually, but unjustly, known as the Langmuir equation.

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the complex is bombarded randomly by water and solute molecules. Occasionally the bonds will be stretched so much that they break, and the drug dissociates. The lifetime of the complex will therefore be very variable. But this variability of the lifetime will *not* be described by the familiar symmetrical Gaussian (or 'normal') distribution; it *will* be described by a probability distribution with a strong positive skew, the shape of the distribution being, in fact, that of a decaying exponential curve (rather than a bell-shaped Gaussian curve). This is the sort of distribution expected for *random* time intervals (see Ref. 2 for further details). If we consider, for example, the case in which the drug concentration is suddenly reduced to zero, we expect the receptor occupancy to fall (towards zero) along an exponential time course with time constant $1/k_{-1}$. Because we are supposing that there is no drug in solution, it follows that whenever a molecule dissociates from a receptor, the receptor can never be re-occupied, so the fact that the occupancy does not instantly fall to zero when the drug is removed simply reflects the fact that drug–receptor complexes endure for a certain, random, length of time before dissociating. Some dissociate soon after removing the drug; some last much longer. When we observe the exponential decline of occupancy we are simply observing the random (exponentially distributed) lengths of time that elapse before each drug–receptor complex dissociates. The time constant, $1/k_{-1}$ (from equation 2) for the exponential decay of occupancy, is simply the *mean lifetime* of the drug–receptor complex (and the half-time for decay is the *median lifetime*). This argument is illustrated explicitly below (Fig. 1) for a different, but exactly analogous sort of experiment. Similarly, the length of time for which a particular receptor stays *unoccupied* is also random (exponentially distributed). On average it is $1/k_{+1}x_A$ so, as expected, it is shorter for high drug concentrations than for low concentrations.

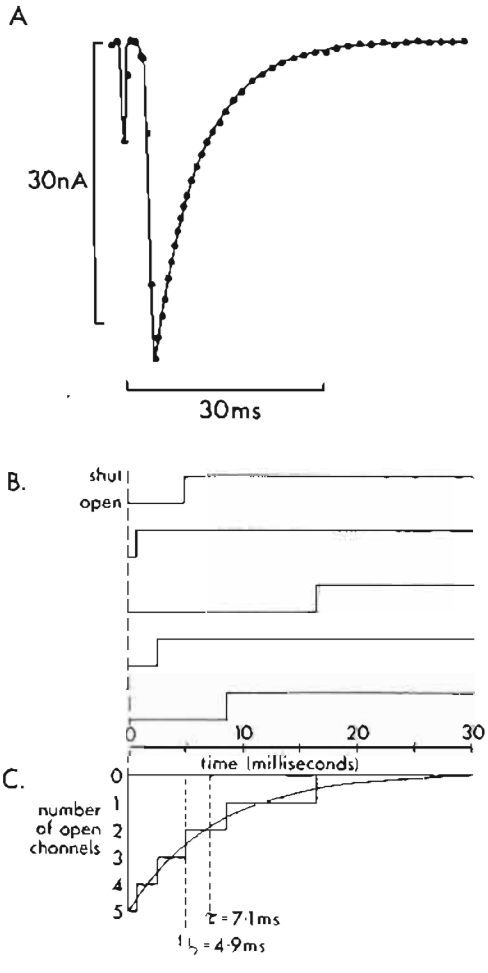


Fig. 1. A. End-plate current evoked by stimulation of nerve (mean of seven). Frog muscle with membrane potential clamped at −130 mV. The continuous line fitted to the decay phase is a simple exponential curve with a time constant of 7.1 ms (reproduced from Ref. 7, with permission). *Inward current is plotted downwards according to the usual convention. About 9000 ion channels are open at the peak.*

B. Simulated behaviour of five individual channels that were open at the time (t = 0) at which the acetylcholine concentration had fallen to zero (opening is plotted downwards). They stay open for random lengths of time (mean $1/\alpha$).

C. Sum of the five records shown in B. The total number of open channels decays exponentially, as is observed (see A), with a time constant $1/\alpha$.

Why concentration jump experiments rarely work

It has been well known for some time that equilibrium constants ($K = k_{-1}/k_{+1}$) for the binding of competitive antagonist drugs can be obtained by the Schild method, or from direct binding experiments (though measurement of equilibrium *agonist* binding has proved much more difficult). It might be supposed that the sort of experiment described above would have allowed the estimation of the rate constants (k_{+1} and k_{-1}) for the association and dissociation, at least for competitive antagonists. But this, unfortunately, is far from being the case. Ever since 1909 people have continued to ask how rapidly drugs interact with receptors, though they almost all (including Hill) got the wrong answers until about 10 years or so ago. The reason for this lack of success lay, in almost every case, in the obstacle posed by *diffusion* through the tissue. The approach originated by Hill assumed that the drug concentration in the solution immediately adjacent to the receptors was *known and constant*. But in fact the rate of diffusion of drug into the tissue has, in most cases, turned out to be a good deal slower than the rate at which the drug interacts with the receptors so the assumptions of the method were contravened and the observed rate of equilibration reflected nothing but the rate of diffusion into the tissue. Of course this problem did occur to many workers; so, in some cases, they checked their results by seeing whether the ratio of the dissociation rate constant and association rate constant, which were inferred from the observed equilibration rate, did actually agree, as they should, with the value of the equilibrium constant (which could be *independently* determined by the Schild method, or from binding experiments). Often quite good agreement was found, and this seemed to suggest that the rates of association and dissociation must be about right. This sounds very reasonable, but unfortunately it is not. The reason lies in the fact that in many cases the rate of diffusion of drug into the tissue is strongly dependent on the extent of the binding of the drug. When a molecule of drug becomes bound, it is no longer free to diffuse towards the centre of the tissue so if a drug can be bound by the tissue it will diffuse more slowly than a molecule that cannot be bound. Furthermore, the extent of this slowing will depend on, among other things, the fraction of the drug that is bound, and hence on the *equilibrium constant* for binding of the drug. Thus it turns out that the diffusion rate of the drug may be dominated by its equilibrium constant for binding so it is not surprising that the

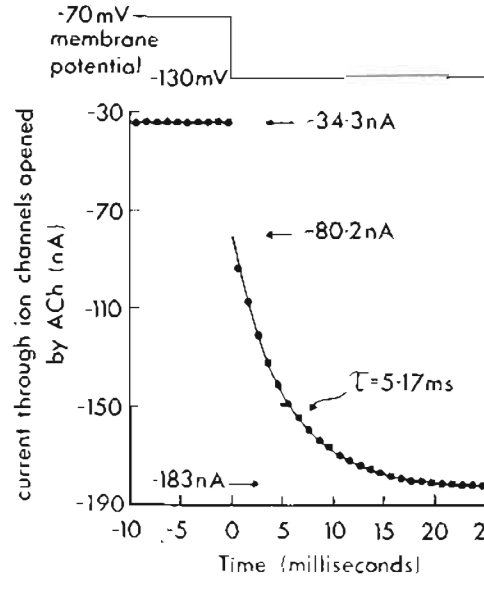


Fig 2 Re-equilibration after a sudden change in membrane potential. Acetylcholine, applied to a frog muscle end-plate, produces an inward current (plotted downwards) of 34.3 nA at a membrane potential of −70 mV. After imposition of a sudden change of membrane potential to −130 mV at zero time (as shown at the top), the current changes gradually to its new equilibrium value of 183 nA. The time course of this re-equilibration has been fitted with a simple exponential curve with a time constant of 5.17 ms. (Colquhoun, Dreyer and Sheridan; unpublished).

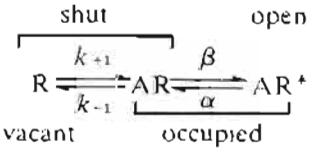
observed rates of onset and offset of the drug can tell us something about its equilibrium constant, even when these rates are quite unrelated to the true rates of association with, and dissociation from, the receptors. Diffusion can mimic the kinetic behaviour predicted by equation 2 with alarming precision, hence the frequent lack of success (see for example, Ref. 6). Other methods are clearly needed.

Methods that do work

During the last 10 years or so, some methods have been developed that really can measure rates. Responses to drugs can be roughly classified into fast mechanisms which equilibrate in a few milliseconds (generally those involved in fast synapses, e.g. the nicotinic acetylcholine receptor or GABA responses), and, on the other hand, slow mechanisms which take more than 0.1 sec to equilibrate (such as responses of smooth muscle or gland cells to muscarinic acetylcholine or β-catecholamine receptors). Curiously enough, much more is known about the rates of the faster responses than of the slower ones: this is because (a) faster responses can be followed by (relatively rapid) electrical measurements (because the effect of the agonist is to open ion channels), whereas the slow ones are often still not slow enough to be followed by the appropriate (much slower) chemical measurements, and (b) the mechanisms of fast responses seem (so far) to be much simpler than those of the slower responses, so observations on fast reactions are easier to interpret.

A simple mechanism for fast responses

A convenient mechanism with which to illustrate many of the principles involved is that first postulated by Castillo and Katz in 1957 for the nicotinic acetylcholine receptor at the neuromuscular junction. An agonist molecule (A) binds to an inactive (i.e. ion channel shut) receptor (R), to form a complex (AR) which may then isomerize to the active state (AR*) in which the ion channel is open.



(3)

Electrical measurements allow us to follow the number of ion channels that are open (AR), though they cannot tell us how the shut channels are divided between the two shut states, R (vacant), and AR (occupied). The chemical (mass action) rate constant for each step is indicated on the appropriate arrow in equation (3). The rate at which this mechanism approaches equilibrium can be derived by standard chemical kinetic methods, but, just as above, it is more enlightening to think in terms of the behaviour of single receptors. For example, the length of time for which an ion channel stays open (its *open lifetime*) will vary randomly, as shown in Fig. 3, with an average duration of $1/\alpha$. Similarly the length of time spent in the occupied but shut state, AR, will*, on average, be $1/(\beta + k_{-1})$.

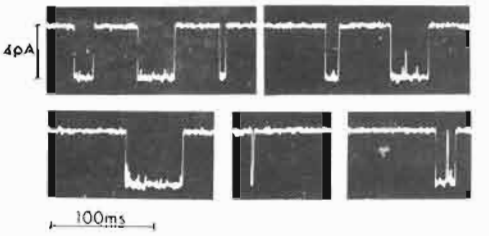


Fig. 3. Examples of the currents that flow when individual ion channels open under the influence of the cholinomimetic agonist suberylcholine (20 nM) (opening is shown as a downward deflection). Notice the constancy of the amplitude (3.6 pA), but great variability of the length of time for which the channel stays open (see text). Two openings occur in quick succession in the last (and perhaps the fifth) events shown. Records are from perisynaptic receptors of frog cutaneus pectoris muscle at a membrane potential of −130 mV (B. Sakmann and D. Colquhoun; unpublished observation).

A special case

The mechanism in (3) postulates that the system can exist in three distinct states. It is, however, commonly believed that the first (drug binding) reaction is very much faster than the second (channel opening) reaction. If this were the case (which is probably not strictly true: see below) then the mechanism in (3) could be simplified to:

$$\text{shut channel (R or AR)} \underset{\alpha}{\overset{\beta'}{\rightleftharpoons}} \text{open channel (AR)} \qquad (4)$$

The true opening rate, β, has been replaced here by the effective opening rate, β', which (unlike β) increases with agonist concentration so $1/\beta'$ is simply the mean lifetime of the shut state†. Just as in the simple binding reaction discussed above, the mechanism in equation (4) would be expected to approach equilibrium along a simple exponential time course: the time constant (half time/0.693) for this exponential will again depend on the sum of the forward and backward reaction rate constants, thus:

$$\tau = 1/(\alpha + \beta') \qquad (5)$$

= mean open time × fraction of channels shut (at equilibrium).

Thus, when the agonist concentration is low (so the opening rate β' is much slower than the shutting rate, α, and the fraction shut is near to one) the time constant is approximately equal to $1/\alpha$, the mean open channel lifetime.

Two types of method

There are two classes of method for measuring rates. These are as follows.

(1) *Relaxation methods.* The obvious method, exemplified above, is to change ('jump') some variable (drug concentration in the example above) so that the system is no longer in equilibrium (it is 'perturbed'), and then to follow the rate at which the system approaches (*relaxes towards*) *its new equilibrium*.

In the case of enzymes, the position of equilibrium can be changed by sudden changes (jumps) in temperature or pressure, (as well as concentration), but these methods are not (yet) feasible for most drug responses. The method of this class that has been most successful is to change the position of equilibrium by a sudden change in *membrane potential* (a *voltage jump*). This, of course, was the method used by Hodgkin and Huxley (long before the rather pompous expression 'voltage jump relaxation' came into common use), for their classical work on axons; examples of its use with drugs are discussed below.

In all of these methods a *sudden* change of concentration (or temperature, or membrane potential) is desirable so that the concentration (etc.) stays constant during the ensuing re-equilibration.

(2) *Equilibrium methods.* The second class of method can be (and usually is) applied when everything is at equilibrium. It sounds odd, at first, that *rates* can be measured when the system is in *equilibrium*. But it is quite clear that this can be done, at least if we can see individual events[11]: for example the lifetimes of individual ion channels can be measured from records such as that in Fig. 3, and the average of these lifetimes can be interpreted (if a mechanism like that in equation 4 is appropriate) as $1/\alpha$. We can therefore obtain an estimate of the chemical rate constant, α, for channel closure. This

* Notice that the mean lifetime of any state depends on the sum of the rate constants for all the pathways by which that state can be left. And the variability of the lifetime will, as in the case of the occupied lifetime discussed above, be described by an exponential probability distribution (see Refs. 4 and 5 for details).

† The effective opening rate is the true rate multiplied by the fraction of shut channels that are occupied (because only the occupied shut channels are *capable* of opening), so, from equation (2), $\beta' = \beta x_A/(x_A + K_A)$. The fraction of channels open at equilibrium is $\beta'/(\alpha + \beta')$.

method works because the term *equilibrium* refers only to the *average* characteristics of the system. If, for example, half the ion channels are open at equilibrium, this does not mean that every ion channel is half open all the time, but that each ion channel is fully open for half of the time, and fully shut for the rest of the time, so an individual ion channel is, in a sense, *never* at equilibrium (indeed the term is not really defined for single channels).

In Fig. 3 only one channel is open at a time; if many are open simultaneously, individual openings and shuttings may not be distinguishable, and the record will merely look noisy as a result of the fact that the number of open channels is not the same from moment to moment. But similar information can still be extracted from this *noise* by the methods discussed below.

These various methods will now be illustrated.

The methods in practice

(1) *Re-equilibration after a sudden change of concentration (concentration jump relaxation).*

The reason why attempts to produce concentration jumps rarely work were discussed above. One case in which this method did, almost certainly, work was described by Schwarz, Ulbricht and Wagner in 1973. They were able to perfuse single Ranvier nodes of myelinated axons so fast that the concentration of drugs at the site of action could be changed within a second or so. They found that tetrodotoxin dissociated from the sodium channels in the node with a time constant of about 70 seconds, much longer than the time for removal of tetrodotoxin from solution. Therefore k_{-1} could be estimated as $1/(70 \text{ sec}) = 0.014 \text{ sec}^{-1}$.

An ingenious method of producing a rapid concentration change has been used by Lester[10]. He used a light-activated agonist, the concentration of which could be rapidly changed by a very brief flash of light.

Another way to produce a rapid change in the concentration of agonist is to use acetylcholine released from a nerve ending (either spontaneously or by nerve stimulation). The acetylcholine concentration in the synaptic cleft rises quickly to a peak, ion channels open, but then, under some conditions, the acetylcholine disappears (because of hydrolysis and diffusion) very rapidly so that shortly after the peak response, there is no acetylcholine left[1]. Thus, to a fair approximation, there is a jump in acetylcholine concentration from a high value to zero, so useful kinetic information can be obtained from the rate at which ion channels close after nerve stimulation, or spontaneous release of a quantum of transmitter. Fig. 1B shows schematically the history of five individual ion channels that were open at zero time (it does not matter, for our purpose, when they originally opened; see Ref. 2). They stay open *on average*, for a time $1/\alpha$, the mean lifetime of the open ion channel. But individual channels stay open for a randomly variable (exponentially distributed) length of time, and, because the agonist concentration has fallen to zero, once a channel shuts it can never re-open. Therefore the total number of open channels, shown in Fig. 1C, is expected to decay exponentially (with a time constant $1/\alpha$) until all are shut. This, as illustrated in Fig. 1A, is just what does happen. And therefore, insofar as the theory (based on a mechanism like that in equation 4) is correct, the decay rate of end-plate currents gives an estimate of the mean open channel lifetime*, and hence of the shutting rate constant, α.

(2) *Re-equilibration after a sudden change in membrane potential (voltage jump relaxation).*

The opening and shutting of ion channels in axons is controlled by membrane potential. But at chemical synapses it is agonists that control the opening and shutting of ion channels. However, even drug-operated ion channels are usually somewhat sensitive to membrane potential, as well as to agonists, so a sudden change in membrane potential can change the position of equilibrium; for example a fixed acetylcholine concentration opens more channels when the end-plate is hyperpolarized than when it is depolarized. This is illustrated in Fig. 2, which shows the current that flows through ion channels opened by acetylcholine in frog motor end-plate. At equilibrium the inward current is 34.3 nA (at a membrane potential of −70 mV). Then the membrane potential is changed suddenly to −130 mV. There is an *immediate* increase in current to 80.2 nA (because there is more voltage to drive it through the channels that are already open). Then comes the interesting bit; there is a gradual increase (which follows a simple exponential time curve) in the current as more ion channels open, until eventually the equilibrium current (183 nA) at the new membrane potential (−130 mV) is reached. If we interpret the result in terms of the mechanism in (4), the time constant (5.17 millisecs in Fig. 2) for the experimental re-equilibration must simply be (from equation 5) $1/(\alpha + \beta')$. So, in principle anyway, such measurements (at various agonist concentrations) can give information about α, β and the equilibrium constant for agonist binding (see, for example, Refs 13 and 14). And, as before, at low enough agonist concentration, the time constant will be close to the mean open channel lifetime.

(3) *Observation of single ion channels.*

Channels opening under the influence of suberylcholine are shown in Fig. 3. As described earlier, the mean channel lifetime can be measured directly from such records. However, although each current originates from one ion channel only, it is not necessarily the *same* channel that opens each time an opening is seen on a record like in Fig. 3. Therefore the average interval *between* openings (which is, in principle, even more informative than the open periods) cannot be interpreted easily except under special conditions where it is known that every opening originates from the *same* channel (see Ref. 15), in which case the mean interval between openings gives an estimate of $1/\beta'$ (see above).

As well as giving information about kinetics, single channel records also show

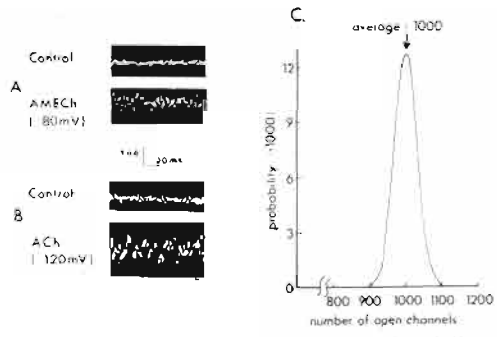


Fig. 4. Fluctuations of the current through rat diaphragm end-plate, in the absence of any agonist (top) and in the presence (below) of agonist, as follows.
(A) acetylmonoethylcholine (at a membrane potential of −80 mV). The fluctuations have a standard deviation of 0.20 nA, and were superimposed on a response (mean current) of 27 nA. At equilibrium about 13,000 ± 190 (2 standard deviations) were open.
(B) acetylcholine (at a membrane potential of −120 mV). The fluctuations have a standard deviation of 0.36 nA, and were superimposed on a response (mean current) of 64 nA. At equilibrium about 21,000 ± 230 channels were open. (Reproduced from Ref. 16, with permission).
(C) The predicted variability in the number of open ion channels. Calculated from binomial distribution for the case where there are one million channels, each with a chance of 1 in a 1000 of being open, so the number of channels that are open on average (i.e. at equilibrium) is 1000 (see text), with a standard deviation of 31.6. For about 5% of the time the number that is open deviates from the average by more than two standard deviations (i.e. is less than 937, or more than 1063).

* With greater formality (and less illumination) this follows from equation 5 because the opening rate, β' is zero if there is no agonist present.

directly the amplitude of current pulses (about 3 pA), and hence the conductance of a single open ion channel, which is commonly about 30 pS (i.e. resistance about 33 000 megohms; one Siemen = one reciprocal ohm). This is information that cannot be obtained by the 'relaxation' methods though it can be estimated from noise analysis.

(4) *Observations of noise, or fluctuations, in the response to agonists.*

When many ion channels are simultaneously open, the individual step-like transitions shown in Fig. 3 may (depending on the recording method) not be distinguishable. But they are there, and they give rise to the noisiness of the record in the presence of agonists shown in Fig. 4A and B.

It has already been mentioned above that the term equilibrium refers only to *average* behaviour. Imagine that there are one million ion channels in the experimental preparation, and that we apply enough agonist to open, at equilibrium, one channel in a thousand. Therefore at equilibrium there are, on *average*, 1000 ion channels open. But *all* of the channels are opening and shutting at random and there is not *exactly* one thousand open at any particular moment. Suppose that an instantaneous picture could be taken of all the channels at a particular instant, and the state (open or shut) of each one noted. This would be exactly analogous to tossing a penny a million times and noting, each time whether it came down 'heads' (channel open) or tails (channel shut) (In this case it is a very biased penny, that comes down heads only once in 1000 throws on average.) Clearly every time the experiment was repeated (an instantaneous picture taken, or a million tosses performed), we would not expect to see *exactly* 1000 open channels (or to get exactly 1000 'heads'). The problem can be treated by standard coin tossing theory*, and the expected distribution of the number of open channels is shown in Fig. 4C. It has a standard deviation of 31.6 and a roughly Gaussian form. So we expect that 95% of the time, the number of open channels will be within two standard deviations (i.e. 63.2) of the mean and we should therefore say, not that there are 1000 channels open at equilibrium, but that there are 1000 ± 63.2. These moment-to-moment fluctuations (of about ±6 percent in this example) in the number of open ion channels are quite big enough to see, and they are what gives rise to the fluctuations, or noise, shown in Fig. 4A, B[9].

How, then, can useful information be extracted from the rather unpromising-looking signals shown in Fig. 4A, B? There are two things that we can measure, the amplitude of the noise, and its frequency. Let us deal with these in turn.

Noise amplitude. A conventional way to measure the amplitude of a fluctuating current (e.g. the alternating mains supply) is to specify the rms (root mean square) current, which is nothing other than the standard deviation of the current, calculated in the ordinary way from a series of values of the current measured at different instants. This amplitude (standard deviation) is, for example, clearly bigger for the record in Fig. 4B than for that in Fig. 4A. The standard deviation of the current is a direct measure of the standard deviation of the number of open channels which was discussed above. If we divide the square of the observed standard deviation by the mean current, we obtain an estimate of the current through a single ion channel (around 3pA) and hence an estimate of the channel conductance (as long as few channels are open and a mechanism like equation 4 is valid†).

Noise frequency. It is obvious by eye that the noise in Fig. 4A contains higher frequencies than that in Fig. 4B. It is therefore natural to ask whether anything interesting can be learned from the frequency, and, if so, how it can be measured. The answer to the first question is yes; we can learn about the rate at which channels open and shut. The answer to the second question follows. Fig. 5A shows, schematically, the opening and shutting of five individual ion channels (mean channel lifetime = 5 ms, say), and Fig. 5B shows the sum of these records. The fluctuating record in Fig. 5B is just the sort of noise illustrated in Fig. 4A,B (though the individual steps are too small to be distinguished in the latter). Now at time zero it is seen that there are four ion channels open (channels number 1, 2, 4 and 5). If we look a bit later on, say 1.0 ms later (as marked in Fig. 5B), there are still four channels open; not surprisingly, they are the same four as were open originally, because, in a short time like 1.0 ms it is quite likely that none of these channels will have shut yet (and that no others will have opened). It is therefore to be expected that the current at any given moment will be very similar to the current at a short time later if, by short, we mean *short relative to the channel lifetime.* In other words the current at any given moment will be highly correlated with the current 1.0 ms later. If we calculate an ordinary correlation coefficient between these two quantities we expect that it will be very good, i.e. near unity, as shown in Fig. 5C. Now consider what happens if, rather than waiting 1.0 ms, we wait 15 ms (see Fig. 5B). This is quite long compared with the mean open

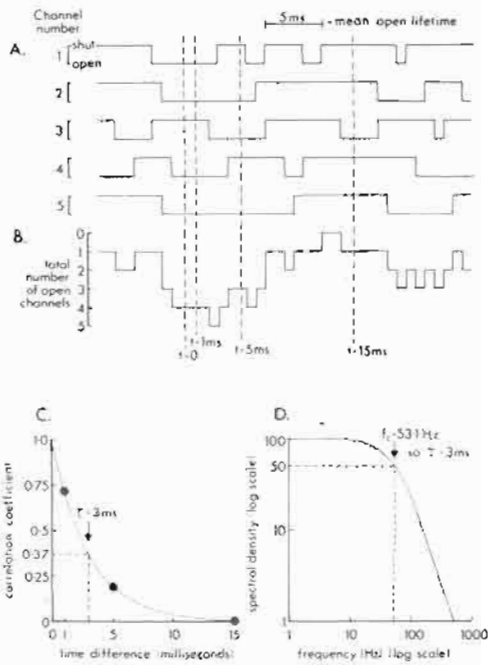


Fig. 5. Explanation of the analysis of the frequency characteristics of fluctuations in the current elicited (i.e. in the number of ion channels opened) by agonists.

A. Simulated behaviour of five individual ion channels (opening is plotted downwards). They are opening and shutting at random with a mean open lifetime of 5 ms.

B. Sum of the five records shown in A. The total number open (and hence the total current) shows fluctuations of the sort that give rise to observations like those in Fig. 4A,B. An arbitrarily chosen line marks zero time, and the times 1, 5 and 15 ms later are also marked with vertical lines.

C. The correlation analysis of noise. Two observations separated by 1 ms are likely to be highly correlated (as shown by the dot). The correlation will be less when the separation is 5 ms, and there is hardly any correlation with a separation of 15 ms. The correlation (according to the mechanism in equation 4) dies out along the exponential curve shown, with a time constant of $\tau = 1/(\alpha + \beta')$. The time constant is 3 ms in the example shown; it is less than the mean open channel lifetime (5 ms), because, as is clear from A, the channels are open for a substantial part of the time. (From equation 5 it follows that the equilibrium fraction of shut channels is 0.6, of open channels is 0.4, $\alpha = 1/(5\ ms) = 200\ s^{-1}$ and $\beta' = 133.3\ s^{-1}$: thus $\tau = 1/333.3 = 3$ ms).

D. Presentation of the same analysis of noise as a power spectrum. The ordinate is normally in units of Amp²/Hz, but is given in arbitrary units here. The amount of noise is halved (from 100 to 50) at 53.1 Hz, so $\tau = 1/(2\pi \times 53.1) = 3$ ms, exactly as found in C.

* The binomial distribution with $N = 10^6$ channels, $p = 0.001$ (probability of being open), gives the mean number of open channels as $Np = 1000$, with standard deviation $\sqrt{Np(1-p)} = 31.6$.

† Under these conditions $Np(1 - p) = Np$ so the variance of the total conductance is approximately $\gamma^2 Np$ (where γ is the conductance of a single channel) whereas the mean conductance is γNp. Their ratio is simply γ.

lifetime, so it is not surprising to see that all the channels that were originally open have shut in the intervening 15 ms, and some others have opened (or the same ones re-opened). Clearly there is no reason to suppose that, just because the current was above average, at zero time, it will still be above average after 15 ms. It could be anything; in other words if we calculate the correlation between the current at a given moment, and the current a long time (relative to the channel lifetime) later, we expect to find a low correlation, as plotted in Fig. 5C. Naturally, at intermediate times comparable to the channel lifetime (5 ms in Fig. 5B), we expect some of the original channels to be still open (e.g. channels 2 and 5 in Fig. 5A), and others to have shut. We therefore expect an intermediate degree of correlation as shown in Fig. 5C. When a graph of correlation (usually called autocorrelation in this context) is plotted against time interval, as in Fig. 5C, the reader may well not be surprised, by this time, to find that theory (for mechanisms like equation 4) predicts that the graph will have the form of an ordinary decreasing exponential curve, and that the time this curve takes to decay is related to the mean open channel lifetime. In fact, Nature, with incredible elegance, has contrived that the time constant for this decay is simply $1/(\alpha + \beta')$, exactly the same value (see equation 5, and above) as found from measurements of re-equilibration.

But, the reader may comment, correlation graphs, like that in Fig. 5C, are only rarely seen in experimental papers. This is, perhaps sadly, true. There is another, and more common way of plotting the analysis of the frequency of noise. It is exactly equivalent to the correlation graph just shown and may be regarded simply as a transformation of it (a bit like transforming an exponential curve by plotting it semi-logarithmically); rather than plotting time along the abscissa, reciprocal time (i.e. frequency) is plotted. And the ordinate reasonably enough, gives the amount of noise* at each frequency. Such a graph is shown in Fig. 5D. It is called a power spectrum (or, better, as a spectral density function). The curve drawn in Fig. 5D is called a Lorentzian curve; it is merely the equivalent (on a spectral density graph) of the exponential curves shown in all the other methods of analysis so far. The graph is flat at low frequencies, but eventually bends downwards. And, clearly this bend would be expected to occur at higher frequencies for noise such as that in Fig. 5A than for that in Fig. 5B, because the former clearly contains wobbles of higher frequency than the latter. The position of the bend is usually measured by the *cut-off frequency* (f_c), that is, the frequency at which the amount of noise is half that at low frequencies. As would be expected from the equivalence of the presentations in Figs. 5C and D, this frequency is very simply related to time constant, τ, found from Fig. 5C (by the relationship $\tau = 1/2\ \pi f_c$). Both analyses of noise frequency, if interpreted according to mechanism (4), give us a value for $1/(\alpha + \beta')$.

* More precisely, it gives the variance (standard deviation squared) of the noise at each frequency. It could be found (approximately) by feeding the noise into a filter that was set to pass only frequencies between, say, 9.5 and 10.5 Hz. The standard deviation of the signal coming out of the filter would be squared and plotted against 10 Hz on the abscissa. If this were repeated with different filter settings a curve like that in Fig. 5D could be constructed. This description also explains why the area under curve is equal to the variance of the original current.

More complicated cases

So far we have dealt only with the simplest case of the kinetics of a single agonist, the binding of which is rapid (see equation 4).

In fact it is quite likely that binding is not as rapid as required by equation 4; sometimes two or more openings may occur in quick succession during the time the receptor stays occupied (this may be the cause of the multiple openings noted in Fig. 3). If this happens, the interpretation of kinetic experiments gets more complicated.

In the presence of certain antagonists, the simple exponential curves (shown in Figs 1, 2 and 5) become sums of two or more exponentials (and, correspondingly, the power spectrum becomes the sum of two or more Lorentzians). These kinetic changes, which correspond to the occurrence of openings in clusters, have given valuable information about how some antagonists work. Their interpretation proceeds along much the same lines as have just been discussed for simple problems but the details are beyond the scope of this article. An account will be found in reviews (e.g. Refs 3 and 12).

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