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Agonist-activated ion channels

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This paper looks at ion channels as an example of the pharmacologist's stock in trade, the action of an agonist on a receptor to produce a response. Looked at in this way, ion channels have been helpful because they are still the only system which is simple enough for quantitative investigation of transduction mechanisms. A short history is given of attempts to elucidate what happens between the time when agonist first binds, and the time when the channel opens.
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Introduction

My aim in this paper is to discuss ion channels from the point of view of the pharmacologist, rather than attempting to review all that is known about them. The title, agonist-activated ion channels (rather than the, now more common, ligand-gated ion channels), is not accidental. It is intended to emphasise that I shall approach ion channels as an example of the pharmacologist's stock in trade, the action of an agonist on a receptor to produce a response. Looked at in this way, ion channels have been helpful because they are still the only system that is simple enough for quantitative investigation of transduction mechanisms. They are, as a consequence, the only system in which some of the classical ideas about agonist action have been tested and measured. A.J. Clark, a great pioneer of quantitative pharmacology (see Rang, this issue), said

> In the first place, there is no advantage in fitting curves by a formula unless this expresses some possible physico-chemical process, and it is undesirable to employ formulae that imply impossibilities.
> It is a question of finding a few systems so simple that it is possible to establish with reasonable probability the relation between quantity of drug and the action produced … (Clark, 1933).

This prescient statement means, for example, that there is little point in fitting a Hill equation (which implies a physical impossibility) or a polynomial, if your aim is to discover something about physical reality (as opposed to using them for the purposes of interpolation, for example). We need, Clark implied, to start by postulating a physical mechanism and try to fit that to observations. To this day, his "few systems so simple …" are essentially restricted to just one system, the agonist-activated ion channel, which is simple enough for his advice to be followed in a quantitative way. I shall discuss only work that has had some success in trying to follow Clark's advice, a very small subset of the ion channel literature.

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Types of channels

All ion channels seem to be oligomers made of several subunits. The ligand-gated ion channel database (http://www.ebi.ac.uk/compneur-srv/LGICdb/LGICdb.php) now contains sequences of 516 subunits from many species, but there are only three main families, which are distantly related to each other. The 'cys-loop' superfamily contains nicotinic acetylcholine receptors, glycine receptors, 5-HT_3 receptors and GABA_A and GABA_C receptors. These are all made up of five subunits. The glutamate (cationic) receptor family (AMPA, kainate and NMDA) is tetrameric and in some ways more related to potassium channels than to nicotinic receptors. Finally there is the ATP-gated family (P2X), probably trimeric (North, 2002). In some cases, the exact subunit composition of native receptors is still uncertain: for example in GABA_A, P2X and neuronal nicotinic receptors. In the last case, even the physiological function remains obscure (see Brown, this issue; Sivilotti & Colquhoun, 1995).

Of the many neurotransmitter receptors, there are only three on which detailed studies of the activation mechanism have been made. The muscle-type nicotinic receptor was the first, and is still the one about which most is known (Ohno *et al*., 1997; Grosman *et al*., 2000a; Sine *et al*., 2002; Shen *et al*., 2003; Engel *et al*., 2003a; and the work described below). The glycine receptor has been investigated much more recently from the mechanistic point of view (see below) and has turned out to be better for this purpose than the nicotinic receptor. The NMDA receptor has also been investigated, but, for technical reasons, it has turned out to be much harder to extract information from it than from either nicotinic or glycine receptors (for example Wyllie *et al*., 1998; Banke & Traynelis, 2003; Popescu & Auerbach, 2003; Erreger *et al*., 2005; Schorge *et al*., 2005).

There are two other sorts of receptor channels that have had a very detailed investigation. They are agonist-activated channels, though not neurotransmitter activated, the agonists being intracellular, calcium ions and cyclic nucleotides. The large calcium-activated (BK) potassium channel behaves in same ways like a nicotinic receptor, but with calcium ions as agonist (see Magleby, 2003). Cyclic nucleotide-gated channels have also been analysed in great detail (Zagotta & Siegelbaum, 1996; Sunderman & Zagotta, 1999).

Naturally occurring mutations of nicotinic and glycine receptors cause (rare) human diseases. In the case of nicotinic (muscle type) receptors, many mutations have been found that cause slow channel congenital myasthenic syndrome (SCCMS: Vincent *et al.*, 1997; Colquhoun *et al.*, 2003; Engel *et al.*, 2003b). In this condition, end-plate currents are prolonged and this causes damage to the muscle end-plate region (probably because of excessive calcium entry) with consequent impairment of neuromuscular transmission. The time course of the synaptic current is controlled primarily by the length of the bursts of single channel openings that underlie the macroscopic current (see below, and Colquhoun *et al.*, 1997; Wyllie *et al.*, 1998). Mutations that lengthen the burst of openings can occur in many different positions, in any of the subunits. Some work primarily by lengthening individual channel openings, but the predominant effect is often to increase the affinity of the binding step, which results in the channel more often re-opening from the fully liganded shut state, thus producing a longer burst of openings. There are also some cases of congenital myasthenia that have similar symptoms but are caused by 'fast channel' mutations, which cause the end-plate current to decay more rapidly than normal. There are also several different mutations in the glycine receptor that cause hyperekplexia, or startle disease (Lynch, 2004; see also Bowery & Smart, this issue). These are all loss-of-function mutations, so the disease resembles poisoning by strychnine. So far, none of the hyperekplexia mutants have been investigated by single channel methods that can reveal underlying mechanisms, but many have been investigated by macroscopic methods (e.g. Lewis *et al.*, 1998; Schofield, 2002; Lynch, 2004).

Development of ideas about ion channel mechanisms

1950–1969

By the early 1950s, John Gaddum, and especially Heinz Schild, had already dealt very successfully with competitive antagonists (see Colquhoun, 2006). The Schild method for obtaining equilibrium constants for antagonist binding was remarkably successful, even when little was known about mechanisms. It was later shown to be valid under a much wider range of conditions than Schild realised at the time (Colquhoun, 1973).

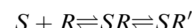
Agonists proved to be much more difficult. Three different, and quite independent, starts were made in the 1950s. The protagonists were Jeffries Wyman (Wyman & Allen, 1951), R.P. Stephenson (Stephenson, 1956) and Bernard Katz (del Castillo & Katz, 1957). Wyman was the first, and arguably the most far-sighted, but he was talking about haemoglobin not receptors. Stephenson attempted the problem without specifying a mechanism, and, as a result, failed (Colquhoun, 1987; 1998; 2005). But Katz was dealing with ion channels, and he postulated a mechanism, which clarified the ambiguities in Stephenson's approach. Of course they were not called ion channels in the 1950s. In their classical study of the end-plate potential, Fatt & Katz commented

> The question arises how such a large flux of ions can be maintained across the presumably minute area of the motor end-plate, and what species of ion are involved. We are not in a position to answer this question,… The only reasonable alternative appears to be that small quantities of acetylcholine alter the end-plate surface in such a way that other ions can be rapidly transferred across … Apparently, we must think in terms of some chemical breakdown of a local ion barrier which occurs as soon as acetylcholine combines with it, and whose extent depends on the number of reacting molecules (Fatt & Katz, 1951).

The shunt resistance that they found during an end-plate potential, 20,000 Ω, corresponds to about a million open channels (of 50 pS each).

In 1957, Katz was addressing much the same problem as Stephenson, the nature of partial agonists (in his case, decamethonium acting at the muscle end-plate). He postulated a simple two-step reaction, which, although oversimplified, could account for the main facts.

> We will suppose, as a working hypothesis, that the 'receptor' y reacts by a similar [to enzymes] two-step process, first forming an intermediate inactive compound which is then changed into an active, depolarising, form (cf. Kirschner & Stone, 1951; Ariens, 1954). We may represent this, for instance, by



$$S + R \rightleftharpoons SR \rightleftharpoons SR'$$

> where SR′ is the depolarising compound whose nature and transformation are, at present, unknown.
> According to this concept, whether a substrate acts as a depolariser or a competitive inhibitor would depend solely on the rate constants of the two steps;

The mechanism is shown in more usual notation in Figure 1.

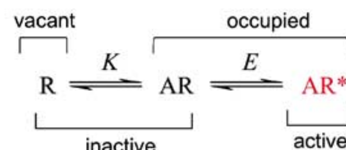


Figure 1 The del Castillo-Katz (1957) mechanism. An agonist, A, binds to a receptor R. The complex, AR can then isomerise to an active state, AR* (in the case of an ion channel, the open state).

Notice that, in 1957, Katz still avoided using the word 'channel', though in the previous year, (del Castillo & Katz, 1956, p. 153) he had used the word in a review.

> Taking a more extreme view, Fatt and Katz (Fatt & Katz, 1951; 1952) suggested that ACh may 'short circuit' the membrane, i.e. create aqueous channels through which small ions can pass without specific distinction

Thus was born the agonist-activated ion channel.

1970–1989

The word 'channel' was not accepted universally until the current that flowed through one channel could be measured.

For the nicotinic receptor, that was achieved again by Katz, whose introduction of noise analysis in 1970–1972 allowed inferences to be made about individual receptors, albeit indirectly (Katz & Miledi, 1970; 1972). This method, especially when used with voltage clamp (Anderson & Stevens, 1973), showed that the current that flowed through an individual channel while it was open was around 5 pA, which corresponds to about 30,000 ions per millisecond. Such a huge flux could be carried only by an aqueous pore.

The first attempts to exploit mechanisms like that proposed in 1957 were by Katz & Miledi (1972), Magleby & Stevens (1972a, b) and by Anderson & Stevens (1973). The methods that were used were all 'macroscopic', that is the measurements were made from large numbers of channels. The kinetic information given by noise analysis or by concentration jumps (in the form of synaptic currents) is, in principle, equivalent (Colquhoun & Hawkes, 1977), and it has turned out to be far more limited than the information that can be found from single channel measurements (see Wyllie *et al*., 1998). This led to problems of interpretation that were impossible to solve in 1972, and this was realised and stated clearly in all of these papers. In the absence of information, the assumption was made that the first (binding) step in the del Castillo-Katz scheme was much faster that the second (gating) step. This implied that openings would occur singly and that the time constants obtained from noise analysis and the time constant for the decay of synaptic currents should both be a measure of the mean open lifetime of the channel. These two measurements were indeed very similar (Anderson & Stevens, 1973), but unfortunately the assumption turned out to be wrong later. This was something that could be tested experimentally only after it became possible to measure single ion channel currents.

When Neher & Sakmann (1976) first showed currents flowing through single nicotinic receptor channels, the field was changed entirely. Single channels had been seen before, in bilayers with antibiotics such as gramicidin (e.g. Hladky & Haydon, 1970), but recording from native channels gave us the opportunity to investigate the physiology of ion channels in much greater detail. Before that could happen, the necessary theory had to be developed to allow interpretation of an entirely new and unfamiliar sort of measurement. Individual molecules behave randomly, so suddenly we had to learn how to deal with stochastic processes. Some of the history of this period has already been recounted by Colquhoun & Sakmann (1998). It was predicted (Colquhoun & Hawkes, 1977) that channel openings would not usually occur singly, but as bursts of openings separated by short shuttings, the whole burst, or *activation*, being what a synapse would see as an 'effective opening', and which would dictate the rate of decay of synaptic currents. This prediction was soon verified experimentally (Colquhoun & Sakmann, 1981; 1985). It is an interesting historical footnote that the interruptions that were first seen in channel openings by Sakmann & Neher in the late 1970s, the *nachschlag* phenomenon, were actually rather rare, and quite long, shuttings, not the much shorter and more frequent interruptions that turned out to be of the greatest interest. These short shuttings were invisible until the giga-ohm seal method was invented (Hamill *et al*., 1981). It was the fine structure of the bursts of openings that allowed the separate estimation of the rate constants for binding of the agonist and for gating of the channel (or, in pharmacological terms, the separate estimation of affinity and efficacy). It provided a solution to the 'binding-gating problem' (Colquhoun, 1998).

A second revolution followed soon after, when the first subunit of the nicotinic receptor was cloned in 1982 (Noda *et al*., 1982). This gave an air of solidity to the receptor – at last we knew what it was made of. Equally importantly, expression of the receptor in oocytes or cell lines now allowed the effect of mutations to be tested easily. The method of single channel recording and recombinant expression were brought together to produce many new results. Three of the first important findings were all the results of collaboration between Sakmann and Numa. First it was discovered that the difference between the foetal and adult forms of the nicotinic receptor (described much earlier by Katz) lay in a difference in their subunit composition (Mishina *et al*., 1986). In the same year it was found that, contrary to some earlier postulates, the M2 transmembrane region lined the ion channel (Imoto *et al*., 1986). And 2 years later, Imoto *et al*. (1988) found rings of charged residues that controlled ion permeation through the channel. It is no coincidence that many of these early spectacular successes concentrated on ion permeation rather than on binding and gating. The latter problem is much harder, but it is the topic of this article, and it is time we got back to it.

Throughout the 1980s the sort of single channel methods used by Colquhoun & Sakmann (1981; 1985) were used to investigate questions like the single channel P_{open} curve and channel block, particularly by Steinbach & Sine (e.g. Sine & Steinbach, 1986; 1987), and by Colquhoun & Ogden (1988) and Gardner *et al*. (1984). The last of these papers showed that the single channel conductance of openings was the same, whichever of a wide range of agonists was used to elicit the openings (contrary to an earlier conclusion from noise analysis; Colquhoun *et al*., 1975). This settled a long-standing question about the nature of partial agonists: it seemed that the open conformation, or at least the pore through it, was much the same regardless of the nature of the agonist.

Other types of channels than the muscle nicotinic receptor began to be investigated, in particular, the ganglionic type of neuronal nicotinic receptor (Colquhoun *et al*., 1989; Ifune & Steinbach, 1990) and glutamate receptors (Cull-Candy & Usowicz, 1987; Gibb & Colquhoun, 1992). Single channel recordings from neuronal nicotinic receptors are hard to make. The channels vanish quickly and their amplitudes are ill-defined, so it has, to this day, not been possible to study them from a mechanistic point of view. Some progress has been made recently on the investigation of mechanisms in glutamate (NMDA) channels (see above), but for various reasons (see Schorge *et al*., 2005) the results are not as clear as with muscle nicotinic receptors or glycine receptors.

1990–present

The 1990s saw considerable advances in knowledge of both structure and function, though rather smaller advances in relating them.

Fitting methods On the functional side, there were big improvements in the methods for making inferences about receptor mechanisms from single channel measurements. Although macroscopic methods (jumps in voltage or concentration, or noise analysis) are undoubtedly useful, the

dissection of single channel mechanisms has almost always required single channel analysis. There are two reasons for this. One is simply that the temporal resolution is much better. It is possible to resolve time constants as short as 10 μs in dwell time distributions about 10 times faster than the best that can be achieved by macroscopic methods. The second reason is that the problem of inference is, in some ways, simpler, in that macroscopic methods require one to cope with the whole matrix of transition rates (the $\boldsymbol{Q}$ matrix), whereas single channel measurements allow one to deal with subsections of it. Furthermore, exploitation of information from the correlations between dwell times provides information about how states are connected to each other of a sort that can never be provided by macroscopic methods (Colquhoun & Hawkes, 1995a, b).

In the 1980s, it was customary to separately fit histograms of open time, shut times, burst lengths and so on with mixtures of exponential probability density functions. The time constants and areas found by this empirical fitting are related only distantly to the rate constants in the underlying reaction mechanism, and only rough corrections can be made for the fact that many events are too short to be detected in most records. Information about mechanisms had to be extracted retrospectively from the overlapping information in such fits. In principle, it has been known since Horn & Lange (1983) that a much better method would be to specify a postulated mechanism in advance, and use it to calculate the likelihood of the entire sequence of open and shut times. The rate constants in the mechanism (which are what we are interested in) are now the free parameters, and they are adjusted to maximise the likelihood. ('Likelihood' is being used in its statistical sense here; it is the probability density of the observations, given some hypothesis about the values of the rate constants.) Furthermore, the problem of deciding how many exponential components to fit vanishes. The calculation of this likelihood was not feasible until some theoretical advances had been made.

First, it was necessary to incorporate information from the fact that adjacent open and shut times are usually correlated. That is so for all the best studied receptors, nicotinic (Colquhoun & Sakmann, 1985; Hatton *et al*., 2003), glycine (Beato *et al*., 2004; Burzomato *et al*., 2004), and NMDA (Gibb & Colquhoun, 1992; Schorge *et al*., 2005). In the 1980s, it was shown that all the information in the record was contained in the joint distribution of open and shut times, and that information about how states are connected could be obtained from correlations (Fredkin *et al*., 1985; Colquhoun & Hawkes, 1987; Blatz & Magleby, 1989). The other problem that had to be solved was how to allow correctly for the fact that short openings and shuttings escape detection. Whenever a short shutting is missed, the opening appears to be longer than it really is, so what we need to calculate the likelihood is not the relatively simple ideal distribution of open times (Colquhoun & Hawkes, 1982), but the distribution of *apparent* open times, which is what we measure from the record (and likewise for apparent shut times). A number of approximate solutions to this problem were proposed (see Colquhoun & Hawkes, 1995b), but an exact solution was found by Hawkes *et al.* (1990; 1992). Once this had been applied to find the *apparent* joint and conditional distributions (Colquhoun *et al*., 1996), the way was clear to do maximum likelihood fits of mechanism directly to (idealised) single channel data. Two programs (both free) are available for doing this sort of fitting, MIL and HJCFIT (see http://www.ucl.ac.uk/Pharmacology/dcpr95.html#hjcfit for a comparison of them). Most work on mechanisms is now done by these methods.

How well do these methods allow identification of mechanisms? Certainly just because a mechanism fits does not mean that it is unique; that is just the normal problem of scientific inference. No inference from the particular to the general can ever be unique. Some mechanisms are not distinguishable even in principle, never mind in practice, and the standard likelihood ratio tests for comparison of two rival mechanisms may not be entirely reliable. Interesting work is being done in both of these areas. In practice, rival mechanisms that fit the data well are compared by a combination of statistical methods, aesthetics, structural plausibility, Occam's razor and, especially, by predictive ability. The development of new methods will, in the long run, reduce ambiguities. For example, if it were possible to measure ligand binding at the single molecule level with the same time resolution as channel opening, much more could be done.

Structural advances Cloning told us about amino-acid sequences of just about every sort of receptor subunit in the 1980s, and, thanks to the pioneering work of Nigel Unwin, information about the three-dimensional structure of the nicotinic receptor began to emerge in the mid-1980s onwards (Unwin *et al*., 1988; Schofield, 2002; Miyazawa *et al*., 2003; Unwin, 2005). It was not until the late 1990s that high-resolution crystallographic structures became available, and there is still no complete high-resolution structure for any ligand-gated ion channel. The only high-resolution structures that exist are for glutamate receptors (both NMDA and AMPA types), and they are restricted to artificial constructs made by linking the two extracellular domains that bind the agonist(s); see Mayer *et al*. (2001), Furukawa & Gouaux (2003); Gouaux (2004). Interesting changes in shape are seen when these constructs bind the agonist, but the fact that the channel itself is absent from them makes attempts to relate these shape changes to function quite speculative. It is, in any case, not possible to be sure whether shape changes are related to pre-opening conformation changes, to opening itself, or to desensitisation, since structure determination is necessarily slow and high agonist concentrations must be used.

No crystal structures are available for any part of the nicotinic or glycine receptors (those for which the most is known about function). For both of these, all the structural information we have comes from Unwin's work on the nicotinic receptor (which has now achieved a resolution of 4 Å; Unwin, 2005), and by analogy with the crystal structure of the snail acetylcholine binding protein. The latter is a soluble protein found in the snail *Lymnaea stagnalis*. It is a pentamer of identical subunits, each containing 210 amino acids. It has only 24% sequence identity with the N terminal end of the human nicotinic (muscle type) α1 subunit (which has 462 amino acids), and lacks the membrane crossing regions, the pore and the large intracellular sequence entirely. Nevertheless, the binding site region shares a strong similarity to that of the muscle nicotinic receptor (Brejc *et al*., 2001; 2002; Celie *et al*., 2004), and the snail protein has been also used as a guide to the extracellular structure of glycine (Laube *et al*., 2002) and GABA receptors (Hosie *et al*., 2003). Unwin's work remains

the only source of information on the structure of the channel and intracellular regions, and on the differences between a shut and an open channel. Attempts to relate structure and function in nicotinic receptors have been reviewed by Colquhoun *et al.* (2003).

How much have we learned from knowing about structure? Structures have certainly made it a lot easier to make plausible hypotheses and to guess which mutations should be made to test them (though this procedure always runs the risk of producing self-fulfilling predictions). They have also made it easier to produce plausible *post hoc* rationalisations for results that were not expected (though not to check whether the rationalisations are right). But structures have yet to add much to our ability to *predict* the effect of a mutation, or of a change in the structure of an agonist. It seems that this Holy Grail of pharmacologists is still some way away.

What happens between agonist binding and channel opening?

Pharmacologists are much concerned with signal transduction. In the case of an ion channel, the transduction of the initial agonist binding occurs within one macromolecule, so we are asking what changes occur within the molecule in order to link the binding of the agonist to the opening of the channel. Looked at slightly differently, we are trying to put more steps into the reaction mechanism, between binding and opening, and to identify the structural states that correspond to these reactions. This enterprise is more closely related to protein engineering than to physiology, but it is what needs to be done if we are ever to attain the ability to predict the effects of changing the agonist structure, or the effect of mutations in the receptor.

There is a more immediate reason why attention has turned, in the last 5 years or so, towards what happens between binding and opening. Up to the end of the 1990s there was optimism that sense could be made of the structure activity relationships of agonists, and of the effect of mutations. All we had to do was to separate the rate constants for individual steps, rather than using the crude macroscopic binding and EC_{50} measurements, which allow no such separation. For example, if a mutation affected *only* the binding affinity to the resting state of the receptor, that would provide good evidence that the mutated residue was in or close to the binding site region. That optimism waned somewhat in the face of experiments that were still hard to explain. For example, many mutations in positions that are certainly far from the agonist binding site appear to mainly affect the microscopic binding rates to the shut channel: e.g., αN217K (Wang *et al.*, 1997) and εL221F (Hatton *et al.*, 2003), which are both in or near the first transmembrane region (M1). The most likely reason for this, at the moment, seems to be that the mechanisms that were fitted were insufficiently close to physical reality. In particular, the quantity that was described as the 'microscopic affinity for the resting binding site on the shut channel' was actually the affinity for a state that had already altered conformation substantially. Any reaction that involves a conformation change is, of course, potentially affected by mutations anywhere in the parts of the molecule that move. But if we want a mutation to give information about the binding site, then clearly we must be able to measure the binding rate constants at a stage when any conformation change is still local to the binding site.

There are two main approaches to dissecting what happens between binding and gating. The first approach is to incorporate a pre-opening conformation change into the reaction mechanism and to use the standard fitting methods. The second approach is to use rate-equilibrium free energy relationships (REFER), as pioneered by Grosman and Auerbach (Grosman *et al.*, 2000b). Each of these will be described next.

The fitting approach

In this approach one postulates a mechanism that includes a conformation change that occurs before the channel opens. This was performed (see Rothberg & Magleby, 1999; mechanism XII) as one way to account for the 'flickering' seen in single channel recordings from large conductance potassium (BK) channels that are seen at saturating concentrations of the agonist (the agonist in this case being intracellular calcium ions).

For neurotransmitter-activated channels, the only case so far in which this approach has proved feasible is for glycine receptors (Burzomato *et al.*, 2004). The glycine receptor has proved particularly suitable for this approach. It has three glycine binding sites and it can open when only one or two of these sites occupied, as well as all three. The concentration dependence of the single channel properties, first shown by Twyman & Macdonald (1991), is spread over a wide concentration range, thus giving information about the number of agonist molecules that are bound. In contrast, nicotinic receptors show monoliganded openings only at very low agonist concentrations (Colquhoun & Sakmann, 1981), and NMDA receptors show virtually no concentration dependence at all, which makes them hardest of all to fit (Schorge *et al.*, 2005).

For the glycine receptor, the minimum mechanism would have four shut states (with 0, 1, 2 or 3 glycine molecules bound) and 3 open states (the lower two rows in Figure 2a). But four shut states are not enough to give a good fit over the whole concentration range (analogous results have been found with the nicotinic receptor and the BK channel). In the past, it has been common to add extra shut states, distal to the open state, in order to get a better fit (Jones & Westbrook, 1995; Rothberg & Magleby, 1999; Salamone *et al.*, 1999; Hatton *et al.*, 2003). The mechanism in Figure 2a has three extra shut states, in the manner proposed for $GABA_A$ receptors by Jones & Westbrook (1995). It has 18 free parameters, all of which are more or less well defined in a simultaneous fit to recordings at four different glycine concentrations (values of the rate constants are shown in the figure). Although this mechanism fits the data well (Burzomato *et al.*, 2004), there are some things about it that are deeply unappealing, if only aesthetically. Firstly, there is no independent reason to believe in the existence of the D states – they are added arbitrarily to get a fit (contrary to the approach advocated by A.J. Clark, quoted at the start of this article). Secondly, in order to get a good fit, it is seen (Figure 2a) that the results imply a strong interaction between one binding site and another (what is commonly, but unhelpfully, called 'cooperativity of binding'). In terms of equilibrium constants, the first binding has low affinity ($K = 14,000\,\mu$M), but once one site is occupied, the next

binding is much higher affinity ($K = 200\,\mu$M), and when two are already occupied, the third is even higher affinity (($K = 10\,\mu$M). This implies that one site can detect when another is occupied, despite the fact that the sites are a long way apart, and the channel is supposed to have not yet undergone any major change of conformation. Both of these objections to the mechanism in Figure 2a are removed by the 'flip' mechanism in Figure 2b. In the flip mechanism there are three 'extra' shut states also, but they are between the resting state and the open state, so they represent a (concerted) change in conformation that occurs after the agonist binds but before the channel opens. In structural terms, it might be imagined (but is not demonstrated) that this represents the 'domain closure' seen in crystal structures (Jin *et al*., 2003). This formulation is precisely along the lines that Wyman & Allen (1951) originally postulated for haemoglobin. The binding sites do not interact, but behave independently (on any one conformation). So the affinity is the same ($K = 520\,\mu$M) for the first, second and third binding to the resting conformation. For the flipped conformation (F), the same is true; the affinity is the same ($K = 8\,\mu$M) for each binding. But the affinity for the flipped conformation is 65 times greater than for the resting conformation, so binding favours the higher affinity F state, and hence activation of the receptor. This mechanism fits the data essentially as well as that in Figure 2a, despite having four fewer free parameters. From the point of view of affinity and efficacy, we see that there are now two different affinities, but only one of them tells us about the resting state of the receptor, so $K = 520\,\mu$M is the 'affinity' in the sense that Stephenson originally intended (but failed to measure). The efficacy now involves two different steps, flipping and opening. Although the gating constant still increases with the number of agonist molecules bound, it does not increase as much as the flipping constant (65-fold increase for each ligand bound). So, according to this interpretation, flipping (while shut) is more important for determination of efficacy than the opening reaction itself. It will be interesting to see how partial agonists behave when interpreted in this way. This case provides a good example of the ambiguities (mentioned above) that can arise in identifying a reaction mechanism. It is important to remember,

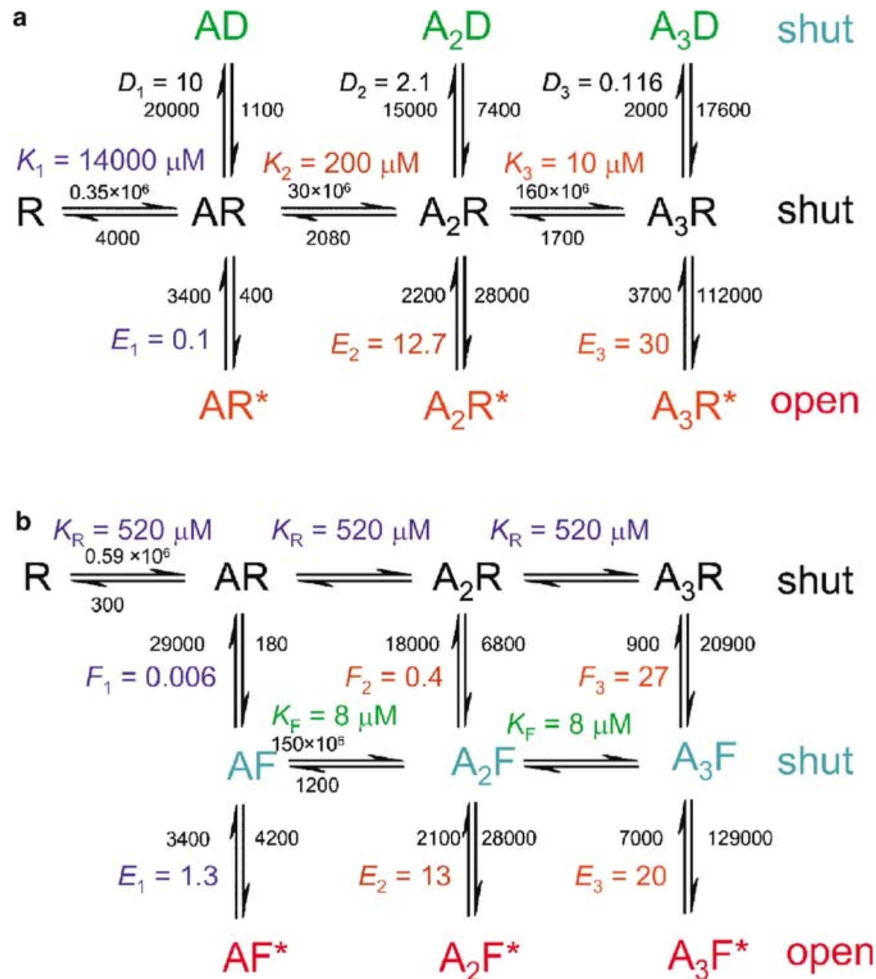


Figure 2 Two possible mechanisms for activation of the glycine receptor. Both mechanisms fit the single channel observations, but they have quite different physical (structural) interpretations (Burzomato *et al*., 2004). Both mechanisms have three open states, and four resting shut states (R), with 0, 1, 2 or 3 glycine molecules bound. Both mechanisms require more than four shut states to fit well. In (a) three short-lived shut states (denoted D) are added, arbitrarily above the resting states. In (b) the flip model, the extra shut states (denoted F) precede opening, so they represent a conformation that can be entered after agonist binding but before opening. The values for the rate constants (found by HJCFIT) that fit heteromeric glycine receptors are shown in small type on the arrows, and the equilibrium constants calculated from them are shown in larger type.

though, that it is not just a matter of whether you get a better fit with the extra shut states placed between resting and open conformations, but it is a matter of deciding what the real physical events are, and how best they can be approximated in a tractable reaction scheme. Otherwise the exercise would degenerate into mere curve fitting.

The REFER approach

The second approach to understanding what happens between binding and gating is based on rate-equilibrium free energy relationships (REFER). This technique has a long history in physical organic chemistry, and has been applied extensively to problems of protein folding (Fersht, 1995; 2000; 2004; Sanchez & Kiefhaber, 2003; Fersht & Sato, 2004; Bodenreider & Kiefhaber, 2005). It was introduced into the analysis of channel gating by Grosman *et al.* (2000b). Auerbach and co-workers in Buffalo have now analysed a heroic number of mutations and the results are very interesting (Grosman & Auerbach, 2000; Grosman *et al.*, 2000b; Cymes *et al.*, 2002; Chakrapani *et al.*, 2003; 2004; Chakrapani & Auerbach, 2005; Zhou *et al.*, 2005). First, an explanation of the REFER method is needed (see also Colquhoun, 2005).

For simplicity, consider only the gating step of a receptor that has all of its agonist binding sites occupied by the agonist. The del Castillo Katz mechanism from Figure 1 is drawn again in Figure 3a, but is now labelled with rate constants. We will consider only the gating step (indicated by the box). In Figure 3a, the opening rate constant is denoted by β, and the shutting rate constant is α, so the gating equilibrium constant is $E=\beta/\alpha$ (E for efficacy). Suppose now that the value of E changes because we make a mutation (or change the membrane potential, or make any other sort of perturbation). A change in E means that either the numerator, β, or the denominator, α, has changed, or both. The extent to which a change in the gating constant results from a change in the opening rate, β, rather than a change in the shutting rate, α, can be judged by plotting $\log(\beta)$ against $\log(E)$ for a series of mutations (or a series of different agonists). This is a REFER plot, and its slope is called φ. If φ is close to 1, this means that the equilibrium constant changes largely as a result of changes in the opening rate constant, β. If the slope is near zero, the equilibrium constant changes largely as a result of changes in the shutting rate constant, α.

Auerbach and co-workers observed that mutations made in the outer part of the molecule, near the agonist binding site, gave φ values close to one (β changes), but as mutations were made deeper, towards the channel gate, φ values decreased progressively towards zero (α changes). Interestingly, this is compatible with the early observation that membrane potential mainly affects α rather than β, because presumably whatever it is that senses voltage must be quite deep so that it is within the electric field. Furthermore, they observed that groups of amino acids that are close to each other appeared to have similar φ values, which was taken as suggesting that these groups of amino acids move together as a 'rigid body' as the channel opens. Although there is room for argument about how constant φ values are within each group of amino acids, and how many such groups there are, it remains true that the systematic change in φ values according to the depth of the perturbations in the channel is very striking. What might this mean in terms of mechanisms? The interpretation of the slope, φ, of the REFER plot in the chemical literature is based on the transition state, the energetic peak that must be climbed to move between the shut and the open conformations. If the transition state resembles the open state, then a value of $\varphi=1$ is expected, whereas if the transition state resembles the shut state, then we predict that φ will be near zero.

Grosman *et al.* (2000b) used a series of different agonists, which gave a φ value close to 0.93, and a very similar value was found for a series of mutations in the binding site region. The fact that these residues are in an open-like state at the receptor's transition state might be taken as a suggestion that this part of the molecule changed shape *before* other parts. This interpretation of transition state analysis in terms of the temporal sequence of events, and indeed the interpretation of fractional values of φ, has given rise to much debate (see Fersht, 2004).

Although there is a certain plausibility in the arguments for interpretation of φ values as an indication of the sequence of events, I have never myself felt that I understood them completely. The arguments about affinity and efficacy needed a concrete example to provide insight into what made sense and what did not, and so does this case. That has been provided by two recent papers that analysed a specified reaction mechanism. It was postulated that there are several very short-lived intermediate states (X_1 to X_5 in Figure 3), which must be passed through in sequence during the conformation change that leads from the shut to the open state. So, for example, the del Castillo-Katz mechanism in Figure 3a would be extended as shown in Figure 3b. In fact attention is focussed on the fully liganded receptor, so the binding step on the left can be ignored.

In a simulation study, Auerbach (2005) showed that changing the rate constants for leaving X_1 produced a large (near 1) value of φ, perturbing the second intermediate in the sequence X_2 resulted in a smaller value of φ, and so on, the smallest values being produced by perturbation of the last intermediate X_5. For this sort of scheme at least, the value of φ *does* have a temporal significance. Zhou *et al.* (2005) took this a step further by finding approximate analytical solutions for the reaction scheme in Figure 3b. Their approximation is good when the two end states (AR and AR*) have much longer lifetimes that all the intermediate states (X_i). This approximation does not hold well with the fast rate of leaving AR that is needed to describe acetylcholine itself (Auerbach, 2005), but it is accurate under the conditions where E is smaller (many of the experiments were done with choline as agonist rather than acetylcholine). The model is undoubtedly oversimplified. REFER plots calculated from it have slopes (φ) that fall from one to zero as E is increased, regardless of which intermediate is perturbed (see commentary by Colquhoun, 2005). And

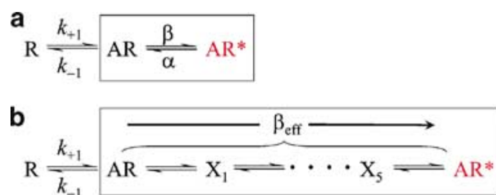


Figure 3 (a) The simple del Castillo-Katz mechanism (A = agonist, R = receptor). (b) Extension by addition of five intermediate pre-opening states. Boxed regions are fully-liganded.

observed REFER plots are more nearly linear than is predicted by the simple theory. Nevertheless, the important point is that, for any given E, the slope, φ, is always lower when the rate constants are changed for later steps in the chain than for earlier ones. The idea that φ is a measure of the temporal sequence of intermediate events now has a concrete basis that heretofore it has lacked. Although one can quibble about the extent to which φ values occur in blocks that define rigid movements of groups of amino acids, the basic idea is now well founded, and has made a major contribution to the understanding of how the ion channel works.

When will we be able to predict the activity of an agonist or of a mutation?

Discussing questions like this is usually a waste of time. But I suspect that this holy grail is still rather a long way away. Many interatomic forces are exquisitely sensitive to separation (sixth power), so even the best resolution of X-ray crystallography may fail to detect important changes in separation. The fact that structure determination has a time resolution of weeks is also a limitation to which no answer is on the horizon at the moment. Molecular dynamics calculations may help, but at present they are restricted to nanoseconds (at best) – who knows when it will become possible to calculate milliseconds. Furthermore, these are theoretical calculations that need experimental verification. It would be of great help if ligand binding could be followed with the same time resolution as channel opening. Fluorescence methods offer hope here, but there is a long way to go before they have the resolution, and especially the length, of single channel recordings. Ion channel people are not likely to be out of a job for some time to come.

Postscript

It may not have escaped the attention of the reader that many of the developments that have been discussed came from Britain (and quite a lot of them from UCL). Langley's original ideas were put into quantitative form by A.V. Hill (1909) when he derived the Langmuir equation nine years before Langmuir (1918). A.V. Hill's time at UCL overlapped with that of A.J. Clark, and it was one of Clark's successors in the chair of pharmacology at UCL, Heinz Schild, who put quantitative experiments with competitive antagonists on a firm footing. Even the originator of ideas about conformation change, Jeffries Wyman, had been a student of A.V. Hill's. Above all, in the field of agonist-activated channels, Bernard Katz's contribution was paramount. He was A.V. Hill's successor and a contemporary of Schild at UCL, and we all owe a great deal to his work and his inspiration (see, e.g., obituaries: Colquhoun, 2003a, b).

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