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# A reinterpretation of mammalian sodium channel gating based on single channel recording

R. W. Aldrich, D. P. Corey & C. F. Stevens

Section of Molecular Neurobiology, Yale University School of Medicine, New Haven, Connecticut 06510, USA

*Some of the traditionally held views about how sodium channels work are shown to be incorrect and a new approach to physical theories of sodium channel operation is suggested.*

OUR notions about the molecular basis for the nerve impulse derive primarily from experimental and theoretical results of Hodgkin and Huxley<sup>1</sup> on the squid giant axon. Their theory is now interpreted as describing the behaviour of a voltage-regulated integral membrane protein, known as the sodium channel, that plays the central role in generation of nerve impulses. Other workers have found that the same general description, with a number of important modifications, is satisfactory for sodium channels of various other preparations. We report here experiments suggesting that, although the Hodgkin-Huxley description is formally adequate, a new interpretation in terms of molecular behaviour is required for at least some mammalian sodium channels.

When the voltage across a resting excitable membrane is changed stepwise to a value sufficiently more positive than the resting level, the permeability of the membrane to sodium rapidly increases and then more slowly declines despite the fact that the membrane voltage is maintained at the positive value that induced channel openings. This increase and subsequent decrease in sodium permeability implies that the sodium channels can progress through at least three distinct functional states: resting, activated and inactivated. Thus, a sufficiently positive voltage causes sodium channels to make the transitions from a (closed) resting state to an (open) conducting state and a (closed) inactivated state, distinct from the resting state. Hodgkin and Huxley identified the rapid increase in permeability with the activation process (progression of channels from resting to activated states) and the subsequent decline with inactivation (entry of channels into the inactivated state). One of their fundamental assumptions was that the two processes are kinetically distinct: activation is rapid and inactivation is slow. Subsequent workers have produced experimental support for this assumption in some preparations by discovering pharmacological agents that appear to remove inactivation while leaving the activation mechanisms unaltered<sup>2–9</sup>.

Our recent studies have shown this assumption, made originally by Hodgkin and Huxley, to be incorrect for at least

some mammalian sodium channels. Specifically, we find that although these mammalian cells exhibit a rapid rise and slow decline in sodium permeability much like in the squid, their sodium channel activation process can be slow and rate limiting whereas inactivation is uniformly rapid. As we describe below, the rate of decline in sodium permeability is not, for these mammalian cells, a manifestation of inactivation time course. Analysis of the molecular mechanisms of voltage dependent gating of course presupposes that these separate processes are correctly identified and characterized.

The response of sodium channels to a step change in voltage is shown in Fig. 1: immediately after the transmembrane voltage is changed from  $-100$  to  $-20$  mV (relative to the cell's resting potential), the sodium permeability (measured here by the probability of a sodium channel being open) rapidly increases and then more slowly decreases. The declining phase has been fitted here by an exponential with a time constant  $\tau_h = 1.9$  ms and the mean open time for individual channels, as will be described below, was 0.5 ms. A traditional analysis would assign the decay time constant  $\tau_h = 1.9$  ms to the inactivation process, and would suppose that activation was complete soon after the peak permeability. Our analysis, presented here, shows that  $\tau_h$  represents a slow component of activation, and that inactivation occurs more rapidly, essentially with a time constant equal to the mean open time of 0.5 ms.

## Experimental methods

We have used the neuroblastoma cell line N1E115 for most of our studies, but have in some instances investigated primary culture of rat myotubes and a pituitary cell line GH<sub>4</sub>C<sub>1</sub>. Single-channel recordings were made using the method originally described by Sigworth and Neher<sup>10</sup> (see also Hamill *et al.*<sup>11</sup>). Most experiments used cell attached patches, but we have also studied channels in the inside-out and outside-out configurations on some occasions. We have found that cell attached patches tend to remain stable for much longer times. Because we used cell attached patches, all voltages specified here are relative to

the resting potential of the cell. Our observations are based on studies of channels in something over 50 patches. Each patch contained from one to approximately 12 sodium channels. We have noticed a tendency for even numbers of channels to occur in a patch; for example, only one patch has had a single channel, whereas two and four channel patches are fairly common and three channel patches are relatively rare.

The measurements we have made are illustrated in Fig. 1. At the top of the figure are seven specimen records of single channel currents. For each record we have determined: (1) the first latency, that is, the time from the onset of depolarization to the first channel opening, (2) the dwell time in the open state for each opening, and (3) the number of channel openings in each trace. In addition, we have averaged groups of individual records together to find the probability of a channel being open as a function of time; a typical result of this averaging process is shown in the lower part of Fig. 1.

## Sodium channel behaviour

We have confirmed the observation that neuroblastoma sodium channels<sup>12-14</sup> behave in a manner typical of other preparations. Figure 2A shows a family of averaged currents obtained for a variety of voltages. Approximately 64 traces were averaged for each voltage displayed. The average currents displayed in Fig. 2A are similar to macroscopic currents studied in other preparations.

Figure 2B shows the conductance at the peak of the current transient normalized to the maximum value and plotted versus voltage. This activation curve has the familiar S-shape. Time constants for the decaying phase  $\tau_h$  are plotted versus voltage in Fig. 2C and show the form of voltage dependence usually found for this parameter. The data in Fig. 2 are characteristic of sodium channel behaviour in all preparations.

## Activation and inactivation

Our goal has been to investigate the inactivation and activation processes separately. To study activation, we have determined the first latency distribution function. This function specifies the probability that an individual channel will open for the first time after  $t$  ms have passed since the onset of a depolarizing step change in voltage. The first latency distribution function describes the probability of first passage time from a starting state into the open state and reflects rate constants between various non-open states including the rate of inactivation of closed channels.

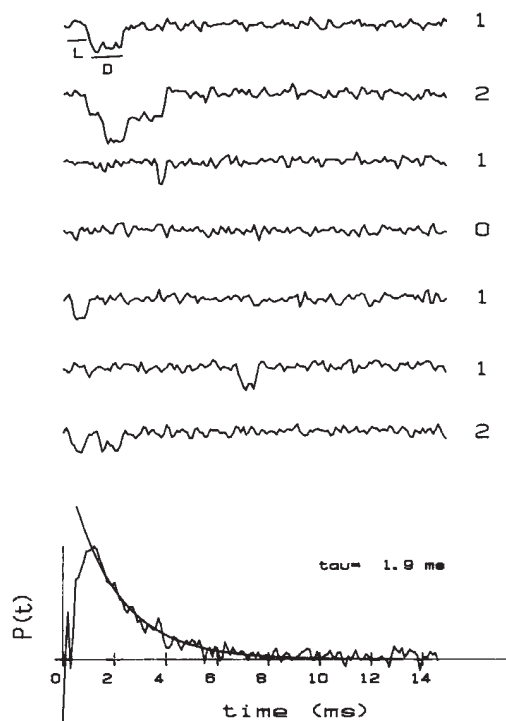
Our investigation of the inactivation rate for open channels begins with a characterization of duration distribution functions (the probability that a channel remains open for  $t$  or more ms). We then consider the question: what fraction of open channels close directly into the inactivated state rather than revisiting a state along the pathway to opening? The rate of open channel inactivation can then be determined, as we shall describe, from mean open duration and the fraction of open channels that close directly into the inactivated state.

In the following discussion we will focus first on the properties of the first latency distribution function and then return to the questions about inactivation and its characterization.

## Latency function

In a patch containing only a single sodium channel, determination of the first latency distribution function is quite straightforward: the patch is depolarized for 15 ms once every second, and the time between the onset of the depolarization and the first channel opening is recorded with an accuracy determined by the sample rate, usually 0.1 ms in these experiments. A cumulative histogram is then constructed which specifies the relative frequency with which first openings occurred later than each sample point.

For a patch containing more than a single channel the determination of the true first latency distribution function is complicated by the fact that the time until first opening depends on not only activation of individual channels but also the number



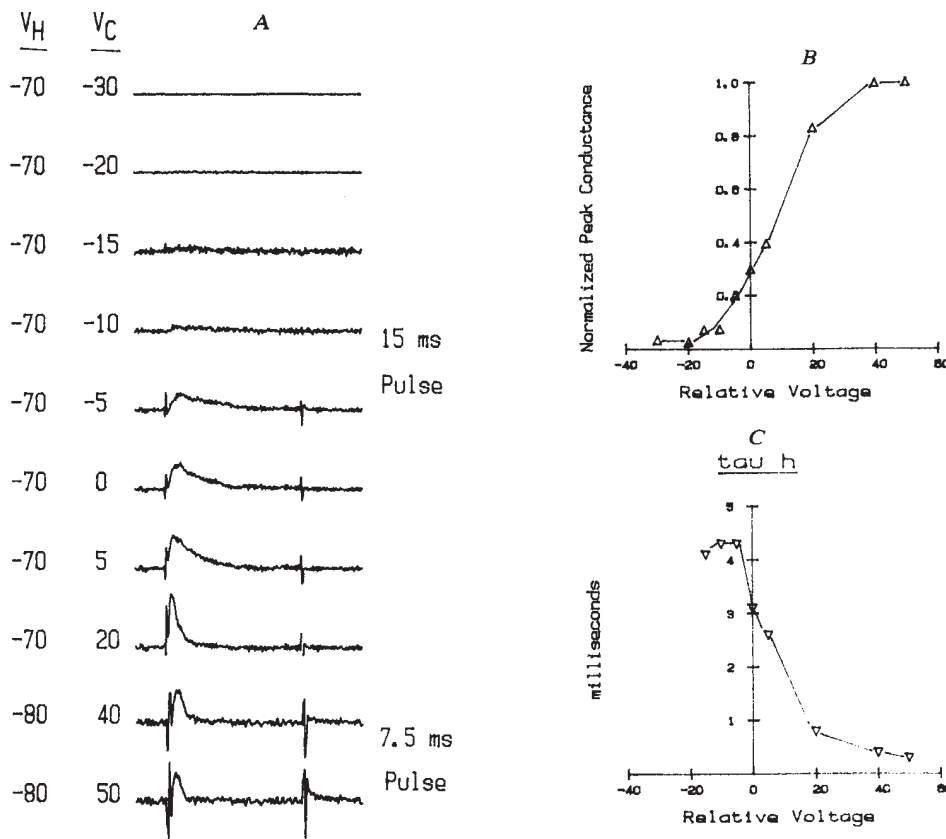
**Fig. 1** Inward currents through single sodium channels. Repetitive depolarizing steps from a holding potential of  $-100$  to  $-20$  mV relative to the resting potential of the cell were applied to a cell attached patch at an interval of 0.8 s. Four sodium channels were present in the patch. Seven representative records are shown to illustrate the variables that were analysed. Leakage and capacitive currents were digitally subtracted. The single channel current is  $\sim 1.2$  pA. Latency until first opening and open channel durations were measured by computer. These are indicated by the letters  $L$  and  $D$  beneath the first record. The number of openings is indicated to the right of each record. Beneath these traces is the channel open probability transient calculated by averaging several hundred records (including the seven illustrated). The falling phase of this transient has been fitted with a single exponential called  $\tau_h$ . It is similar to  $\tau_h$  measurements from macroscopic sodium currents. Durations of opening events that overlap in time were measured by randomly assigning pairs of the opening and closing transitions. For example, when two openings overlapped, the first closing event was paired with the first opening event half of the time and with the second opening event half of the time. The error introduced by this method is small when the patch contains a small number of channels. The currents were filtered at 2 kHz, the temperature was  $9.5^\circ\text{C}$ .

of channels present. If the channels are identical and independent, however, the true first latency distribution can be obtained from the apparent one by simply taking the  $N$ th root of the apparent distribution function where  $N$  is the number of channels present in the patch. The number of channels present is estimated simply by inspecting between 100 and 2,000 records and counting the maximum number of channels open simultaneously; this procedure gives a lower limit on the actual number but is accurate if the number of channels in the patch is no more than about 4 or 5, a sufficient number of large depolarization steps are used, and the holding potential is negative enough so that there is little resting inactivation. We have found this simple counting method seems to work as well as more complicated statistical determinations of channel number.

First latency distributions shown for three different potentials in Fig. 3 contain at least two distinct rate constants (reciprocal time constants) and can be approximately fitted by the function

$$F(t) = (1 - P_0)(R_1 e^{-R_2 t} - R_2 e^{-R_1 t}) / (R_1 - R_2) \quad (1)$$

The rapid and slow rate constants,  $R_1$  and  $R_2$ , present in the first latency distribution both get larger with increasing



**Fig. 2** Voltage dependence of sodium channel kinetics. *A* shows average currents for steps to various command voltages. Voltages are relative to rest potential. The currents can be seen to increase in speed as the membrane is more depolarized. *B* shows the peak conductance for each of these averages normalized to the maximum and plotted against voltage. *C* is a plot of  $\tau_h$  versus voltage. The patch contains at least 9 channels. Filtered at 2.5 kHz. Temperature: 20 °C.

magnitude of depolarization. Note that the asymptotic value  $P_0$  of the first latency distribution is not zero; this non-zero asymptote reflects the fraction of channels that pass directly into the inactivated state without ever opening (see ref. 15).

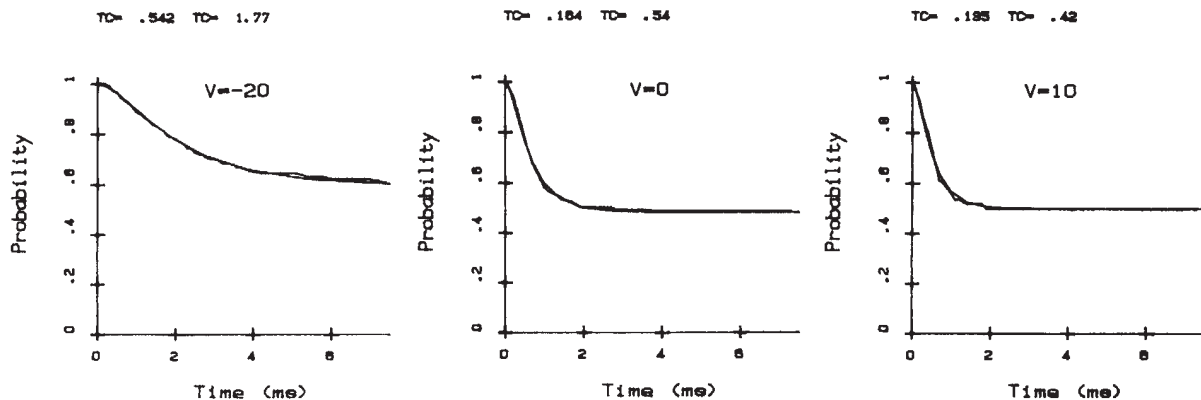
The most important feature of the first latency distribution function is that the smaller rate constant is comparable to the rate for the declining phase of the sodium current, at least over most of the activation range shown in Fig. 2. Many channels are opening for the first time during the process that normally would be described as inactivation. Thus, the activation process takes place throughout the sodium current transient and plays an important role not only in the initial rise to a peak but also in the decline: activation has very slow components.

### Rate of open channel inactivation

A channel, once open, remains open for a random length of time. The duration distribution function  $L(t)$  that describes this behaviour is obtained from single channel current records by measuring the duration for each channel opening and compiling

a histogram that specifies the relative frequency of finding an open time of  $t$  ms or longer. The durations are approximately exponentially distributed and show the interesting feature that, over most of the activation voltage range (see Fig. 2), the mean open times (and in fact the entire distribution function) are independent of membrane potential. Mean open times at room temperature are close to 0.5 ms. Fenwick *et al.*<sup>16</sup> have reported a decrease in mean open duration at low depolarizations where there is little activation. We have also found shorter durations at depolarizations where the probability of a channel opening is low (-30 to -15 mV in Fig. 2). It is clear, however, that over all but the lowest part of the activation range the duration is independent of membrane potential.

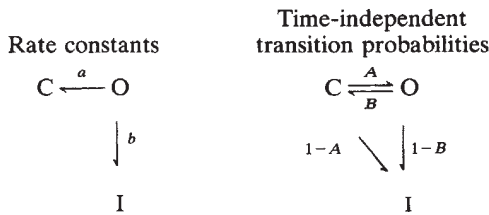
The dwell time in any state is the reciprocal of the sum of the rates for leaving that state. For an open channel, then, the open duration reflects the rate of returning to a state visited prior to the opening and also the rate of inactivation. Specifically, if  $a$  is the rate of revisiting the opening pathway and  $b$  is the rate of progressing to an inactivated state (or states), the mean



**Fig. 3** First latency functions at three voltages. These are calculated as described in the text. Superimposed on each is a fit to equation (1) with time constants (reciprocals of the rate constants  $R_1$  and  $R_2$ ) indicated above the curves (in ms). As the membrane becomes more depolarized both of the time constants in the fits become faster. This patch contained four channels. Temperature, 16 °C.

open time is  $1/(a + b)$ ; furthermore, the fraction of closings from the open state into the inactivated state is  $b/(a + b)$ . If we can estimate the fraction of transitions that proceed to the inactivated state, then the rate constant for inactivation can be calculated from this fraction and the mean open time.

We consider three groups of states: resting, open, and inactivated. The channel starts in one of the resting states, we observe entrance into the open state, and we know that channels rarely if at all return from the inactivated state at the membrane voltages that produce openings; the inactivated state is an absorbing one. Consider now transitions between these states (or groups of states) without regarding time; that is, we ask only for the probability of a transition to a particular state, whenever it occurs.



For example, at a particular voltage, the probability of the transition from closed (C) to open (O) may be  $A$ , which would mean that the probability of entering the inactivated state (I) from resting states would be  $1 - A$ .  $1 - A$  can be estimated from the number of records in which a depolarizing voltage is maintained for a sufficiently long period of time and no channel opening occurs. Similarly, a channel in the open state may pass directly to the inactivated state with a probability of  $1 - B$  or it may return to one of the resting states, when the transition occurs, with the probability  $B$ . If we can estimate  $A$  and  $B$ , we can then calculate the rate of inactivation from the mean open time and the relation  $1 - B = b/(a + b)$ .

The situation just described is a Markov chain with an absorbing state (inactivated). Straightforward calculations reveal that, for a single channel, the probability of observing  $k$  transitions into the open state before the channel enters the absorbing (inactivated) state  $P(k)$  is given by

$$P(k) = P_0(O) \left( \frac{1 - AB}{B} \right) (AB)^k \quad (2)$$

where  $P_0(O)$  is the probability of being in state C initially; this probability specifies the resting inactivation. For an  $N$ -channel patch, the probability of observing  $k$  openings per depolarizing step is given by the  $N$ -fold convolution (denoted by  $N^*$ ) of the distribution (equation (2)):

$$P(k)_N = \{P(k)\}^{N^*} \quad (3)$$

We have used these relations to estimate the probability  $1 - B$  that an open channel will pass directly into the inactivated state by observing the relative frequency of  $k$  transitions into the open state for patches with various numbers of channels from 1 to 4.  $1 - A$  has been estimated by the  $N$ th root of the fraction of records with no opening and then  $1 - B$  found by fitting the data by equation (3). A histogram giving the relative frequency of  $k$  transitions per trace for a 4-channel patch is shown in Fig. 4; data in the figure have been fitted with equation (3), and  $1 - B = 0.79$ . In all cases, the probability that a channel makes a transition directly from open to inactivated is between about 0.8 and 1, and the fraction of open channels entering the inactivated state appears not to depend on membrane potential (see Fig. 5). In other words, channels usually close by inactivating at all voltages throughout the activation range (see Fig. 2).

The calculation described above makes several idealizing assumptions which are difficult or sometimes impossible to check directly. For example, we obtain a lower limit for the number of channels in the patch by observing the number simultaneously open, but we cannot be sure the patch does not contain more channels than we suppose. Furthermore, we have assumed that the inactivating state is absorbing, whereas an inactivated chan-

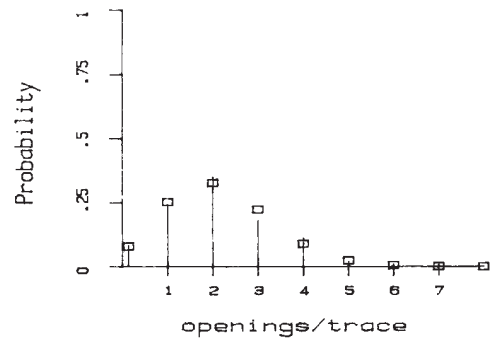


Fig. 4 Histogram of the number of openings per depolarizing step to  $-20$  mV from a holding voltage of  $-90$  mV (relative to the resting potential). Superimposed on the histogram is the solution of equation (3). The probability of a channel opening  $A = 0.47$  and the fraction of openings which closed to the inactivated state  $1 - B = 0.79$ .

nel may occasionally reopen. Also we have neglected the fact that there must be several different resting states and have instead assumed there to be only one. Each of these assumptions, if incorrect, would give an underestimate for the fraction of channels closing directly into the inactivated state. For these reasons the fraction of channels closing into the inactivated state given above is a lower limit of the actual value, and channels must close into the inactivated state at least 80 or 90% of the time.

Since the open channels predominate close to the inactivated state and do not reopen, the mean open time is essentially the reciprocal of the inactivation rate constant. Since the durations are much shorter than  $\tau_h$  at most voltages (see Fig. 5), the time course of the average sodium current must be predominately determined by activation. At any time during the transient, current is flowing through channels that only recently opened for the first time.

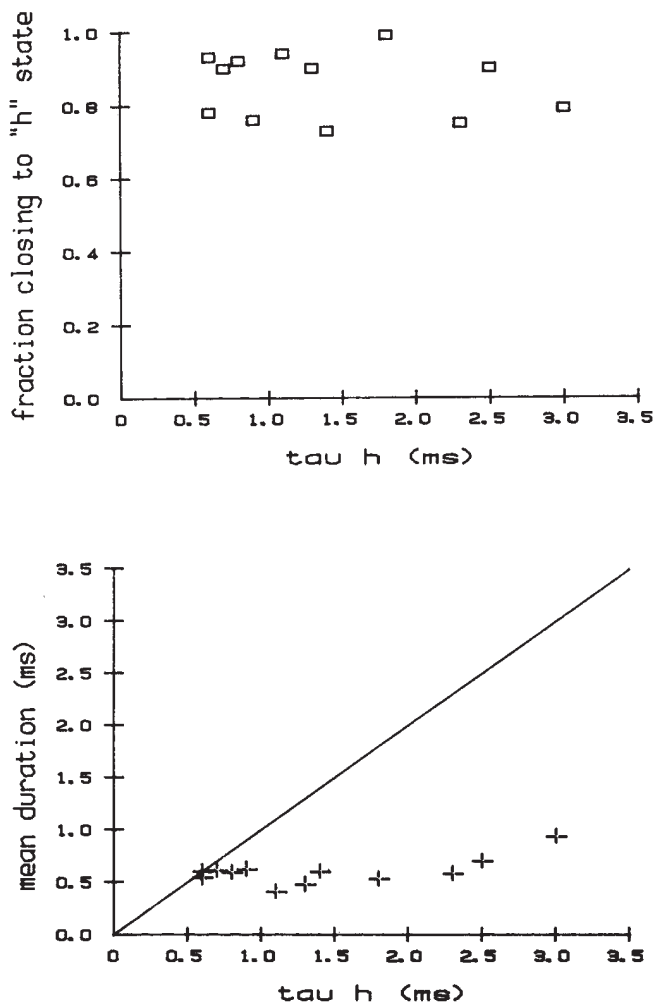
### An alternative approach

We have concluded, then, that activation is the rate limiting step, and that inactivation is rapid and is not significantly dependent on voltage over the voltage range where activation occurs. These conclusions have been reached by examining the distribution of times until first opening, the distribution of open durations, and the probability that an open channel enters the inactivated state when it makes the closing transition. Our conclusion can be checked in another way as indicated by the following argument. The probability that a channel is open at time  $t$  is given by the equation

$$p(t) = \int_0^t f(t - \tau) M(\tau) d\tau \quad (4)$$

where  $p(t)$  is the probability of finding a channel open at time  $t$ ,  $f(t)$  is the probability density for a channel first opening at time  $t$  and  $M(t)$  is the probability of finding a channel open at time  $t$  given that it first opened at time 0. This convolution integral enumerates the ways a channel can open at all times previous to  $t$  and then be found open when observed at time  $t$ . The function  $M(t)$  can be further decomposed into the probability that a channel has not closed by  $t$  and the probability that a channel closed before  $t$  and then was found open when observed at time  $t$ . Insofar as channels usually close directly into the inactivated state, reopenings are negligible and the function  $M(t)$  is just the duration distribution function  $L(t)$  that we have determined experimentally. Because both the functions  $f$  and  $L$  can be measured directly from our data, the convolution of these two functions should reproduce the observed probability of a channel being open if each channel only opens one time. We have tested this equation and found it to be accurate.

Figure 6 shows the convolution of duration and latency distributions superimposed on average currents at three voltages.



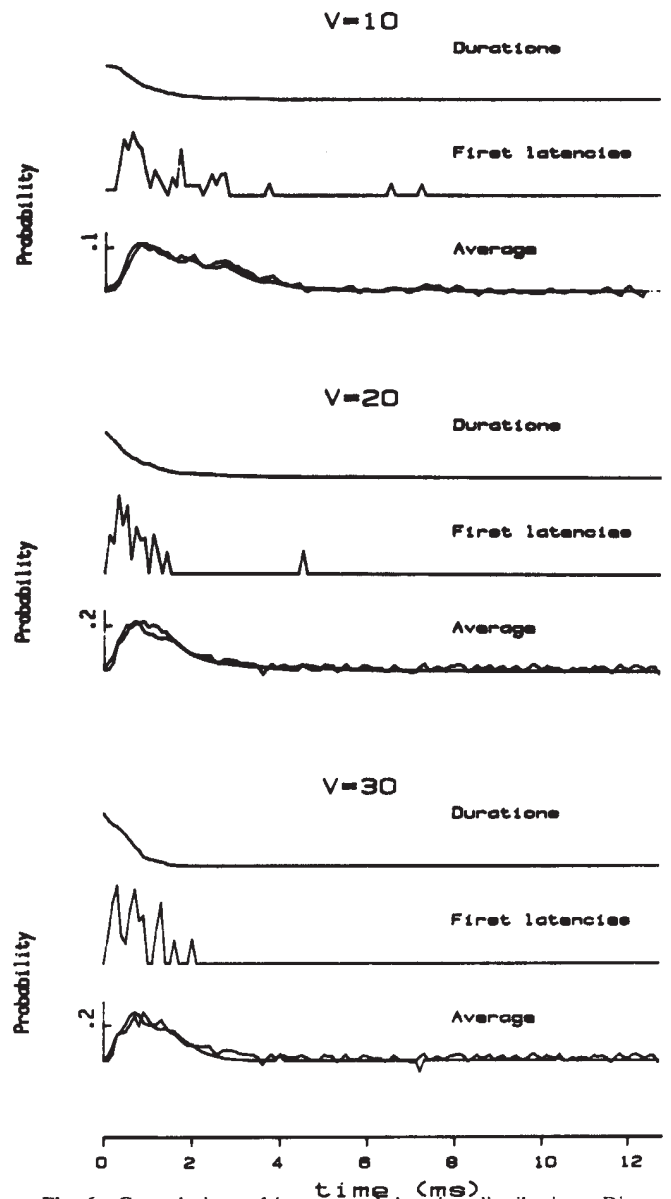
**Fig. 5** The fraction of channels closing to the inactivated state and the mean duration are plotted versus values of  $\tau_h$  from the corresponding average. Data are pooled from many different patches, for various depolarizations.  $\tau_h$  was used for the abscissa instead of voltage to permit comparison of patches from different cells which may have had different resting potentials.  $\tau_h$  can be approximately translated into voltage by using the  $\tau_h$  versus  $V$  curve in Fig. 2. The fraction closing to the inactivated state was calculated as detailed in the text and Fig. 4. The straight line shown in the lower panel would be expected if  $\tau_h$  were determined by the open durations. Temperature ranged from 16 to 21.8 °C.

Agreement between predicted and observed functions is good. If the channels reopened appreciably, the experimental averages would decay more slowly than the calculated ones. Note also that as the average currents get faster with higher depolarizations the duration distributions remain similar but the first latency distributions become faster.

## Conclusions

Our results suggest that channels open only once per depolarization epoch; they primarily close to an inactivated state and do not reopen. The inactivation rate is fast and not very voltage dependent. Slow components of activation are rate limiting except at those voltages for which  $\tau_h$  approaches its asymptotic value (see Fig. 2).

These conclusions are different from the Hodgkin and Huxley interpretation of activation and inactivation. They agree in some ways, however, with more recent work on squid and amphibian axons. In particular, based mainly on the lack of a gating current component with the time course of inactivation, Armstrong and Bezanilla<sup>17</sup> and Nonner<sup>18</sup> have concluded that inactivation is



**Fig. 6** Convolutions of latency and duration distributions. Displayed for each voltage are: duration distribution functions; first latency probability density functions (calculated by taking the derivative of first latency functions such as in Fig. 3); the convolution of these two functions; and the corresponding probability transient (noisier trace). Convolutions were performed by multiplying the Fourier transforms of the two functions and taking the inverse transform of the product. Individual points plotted in this figure are connected by straight lines. The patch contained three channels. Temperature, 14.5 °C.

not voltage dependent (but see ref. 19). They have shown that, at a number of voltages, the time course of sodium current and gating currents can be well fitted by a model in which inactivation rates are not dependent upon voltage<sup>4,18,20</sup>. Our results, for single sodium channels from mouse neuroblastoma, are in agreement with their interpretation.

There is a discrepancy, however, between neuroblastoma and squid and amphibian sodium channels in regard to slow components of activation. Experiments in these tissues with agents that remove sodium channel inactivation, such as pronase<sup>2,3,9</sup> and *N*-bromoacetamide (NBA)<sup>7,8,21</sup> show that activation is essentially over by the time the current reaches its peak. This is in contrast to our first latency results and to single channel experiments on NBA-treated sodium channels from rat myotubes<sup>22</sup>. These authors find that after NBA treatment (presumably removing inactivation), activation continues long after

the time of peak current in untreated channels. These differences may reflect a difference between mammalian and other sodium channels. Mammalian sodium currents have been found in some cases to have much faster inactivation than squid or amphibian channels<sup>23</sup>. If the inactivation rate is fast, peak current will occur at earlier times than if it is slow and the probability of a channel being open at the peak will be less than if inactivation is slower. Consequently, activation will not be complete by the peak. In tissues where inactivation is slow relative to activation (such as squid axon and amphibian nerve) we would expect the channels to open more than once, activation to be mostly over by the peak, and peak probabilities to be relatively high.

One of our central conclusions is that one cannot necessarily identify the rise in probability of the channel being open with activation and the decline with inactivation. Rather, over most of the activation range, the entire process reflects primarily activation, and inactivation is rapid and voltage independent. These observations have obvious implications for underlying mechanisms. If one tries to interpret the molecular basis for sodium channel activity in terms of the traditional activation-inactivation scheme, quantitative if not qualitative errors are certain to result. A specific example of such an error is the suggestion in earlier work from our laboratory that activation and inactivation are independent processes<sup>24</sup>. The arguments made were based on the assumption that the rate limiting step in the decline of sodium channel probabilities was inactivation whereas, under the conditions of that experiment, this declining

phase was dominated by activation. We now find, based on experiments using a three-pulse protocol<sup>15</sup> that inactivation of closed channels is much slower than that of open channels so that the inactivation process must be coupled to activation.

Our conclusions also simplify the task of understanding sodium channel behaviour by decomposing the entire problem into smaller independent problems. For example, earlier workers have been forced to make a theory that accounts simultaneously for the entire activation-inactivation sequence. Because the sodium channel behaviour can be reconstructed by convolving the first latency densities with the duration distribution function, we can attack the overall problem by treating these two functions independently. For example, one may make a theory for the durations without having to worry about the first passage times to the open state. In fact, because durations are approximately exponentially distributed, a reasonable theory is to assume the existence of only one open state with transitions occurring out of the state according to a Poisson process. Furthermore, most of these transitions are to the absorbing inactivated state. Similarly, one can construct theories for the first passage time to the open state without being concerned with the later behaviour of the channel and the possible existence of, for example, multiple open states.

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## Modular arrangement of functional domains along the sequence of an aminoacyl tRNA synthetase

Maria Jasin, Lynne Regan & Paul Schimmel

Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA

*Gene deletions show that much of Escherichia coli alanine tRNA synthetase is dispensable for each of three activities and that these activities appear to require specific domains arranged linearly along the polypeptide. Thus, variable fusions of extra polypeptide domains to a catalytic core may account for the diverse sizes of aminoacyl tRNA synthetases.*

CONSIDERABLE variation in subunit size and quaternary structure is shown by individual members of the aminoacyl tRNA synthetase class of enzymes<sup>1,2</sup>. This contrasts with the similar reaction catalysed by each of the enzymes and by the small size variation shown by the various tRNA species<sup>3</sup>. Quaternary structures which have been characterized include  $\alpha$ ,  $\alpha_2$ ,  $\alpha_2\beta_2$ , and  $\alpha_4$ <sup>1,2</sup>. Subunit sizes range from approximately 330 amino acids for the Trp-tRNA synthetase<sup>4</sup> to lengths over 1,000 amino acids for the isoleucine<sup>5</sup> and valine enzymes<sup>6</sup>. Because the Trp-tRNA synthetase subunit has a complete set of sites for substrates<sup>7</sup>, the core synthetase necessary for achieving aminoacylation appears to require as little as 300-350 amino acids. This suggests that, for larger synthetases, sizeable sections of the

polypeptide sequence are not for the purpose of aminoacylation. Indeed, additional functions have been proposed for some synthetases, such as regulation of gene expression<sup>8</sup>.

These issues were explored further with *Escherichia coli* Ala-tRNA synthetase, an  $\alpha_4$  tetramer which is the largest of the known synthetases<sup>9</sup>. The entire 875 amino acid sequence was determined previously by sequencing of the coding region of the gene and also by independently establishing the amino acid sequence of large sections of the polypeptide<sup>10</sup>. Our purpose was to determine the layout of the functional parts of this polypeptide chain. This was approached by dissecting the polypeptide into pieces and attempting to find, for example, the smallest piece which enables the enzyme to function *in vivo* and