Chapter 13
Analysis of Nonstationary Channel Kinetics

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1. Introduction

Many voltage-dependent channels open transiently when activated by a sudden change in voltage. This transient behavior presents a particular problem in studying channels with single-channel recording because it cannot be studied with the membrane potential held constant. Methods for the analysis of channel opening and closing at a constant membrane potential are described in several other chapters in this volume. In this chapter, we discuss the analysis of single-channel data from experiments in which the membrane potential is suddenly changed and the probabilities of the channel occupying particular conformational states change from one distribution to another. This approach is essential for studying channels that inactivate, but it also complements the usual methods for studying other channels and gives information that is difficult or impossible to obtain otherwise.

A system of ionic channels perturbed from the steady state by a voltage step is an example of a nonstationary system. The following discussion begins by defining nonstationarity and then relates this mathematical definition to the behavior of voltage-dependent channels. A collection of many single-channel records taken under identical conditions forms a statistical ensemble, and averaging channel behavior over such an ensemble describes a time course of channel opening just as a record of macroscopic current through a large number of channels does. The latter part of the chapter explains how ensembles of single-channel records can give more information than macroscopic current records do about the kinetic scheme for a channel. In general, any model for channel behavior must predict not only the time course of channel opening but also the statistics of the openings and closings. Even without a general model, particular questions about channel behavior can be addressed by compiling conditional averages, as illustrated by a specific example with voltage-activated Na⁺ channels.

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2. A Nonstationary Process Has Occupancy Probabilities That Change with Time

A stochastic process is called stationary if the probability of being in each state does not depend on time. Thus, a stationary process will have a mean and variance that are constant. A nonstationary process, on the other hand, will have a time-varying mean and variance. In practice, nonstationary single-channel data can occur in two ways:

1. The occupancy probabilities can change with time because of drift in the experimental conditions or run-down of the preparation.
2. The experimenter can arrange a sudden change in the rate constants for the transitions between states. The occupancy probabilities for the open and closed states will then change with time as the system relaxes to a new equilibrium.

The first of these sources of nonstationarity, slow drift, is an important possible artifact, especially because it takes a long time to collect statistically significant data on a single channel. It should be eliminated as much as possible, and its contribution to experimental results should be evaluated and controlled for. However, when nonstationarity is experimentally arranged, it can yield valuable information about the kinetics of channel gating.

3. Relaxation of Current after a Voltage-Clamp Step Is a Nonstationary Process

The voltage dependence of channels can be studied by observing their behavior during voltage steps. Figure 13-1 shows currents through a Ca\(^{2+}\)- and voltage-activated K\(^{+}\) channel subjected to a sudden voltage change. Each of the four records was obtained when the membrane potential was suddenly stepped to +80 mV after a long time at the holding voltage (-40 mV). When the membrane potential is held at -40 mV, the channel is almost never open, but at +80 mV, the channel is open nearly all of the time. Immediately after the voltage change, the channel has had no time to flip between the open and closed states, so the probability of its being open is the same as it was before the voltage change. After a long time at the new voltage, the probability that the channel is open is, of course, just the steady-state probability at +80 mV. In between the very early and very late times, though, the channel-open probability relaxes from the old steady-state probability to the new. During this relaxation time, the process is nonstationary. If we were to determine the channel-open probability by measuring the fractional open time for the whole duration of the pulse, we would severely overestimate the probability at early times. Since the probability is changing with time, we cannot use methods for the analysis of stationary processes. A method we can use instead is ensemble analysis.
Figure 13-1. Currents through Ca\(^{2+}\)- and voltage-activated K\(^{+}\) channels from cultured pituitary cells (GH). The four single-channel current records were recorded in response to the voltage clamp command at the top. Leakage and transient current have been subtracted. Below is an average of 64 such records, at the same scale. The records are from an inside-out detached patch containing a single channel, bathed in symmetrical 160 mM KCl with 1 μM free Ca\(^{2+}\) (buffered with EGTA) on the inside surface. The probability that the channel is open was computed by finding all of the detectable open and closing transitions, constructing for each record a schematic representation that has a value of 0 when the channel is closed and 1 when it is open, and averaging these idealized records together. The voltage-clamp currents were low-pass filtered at 4 kHz with an eight-pole Bessel characteristic and sampled by a computer at a rate of one sample every 100 μsec. The temperature was 23°C.
4. An Ensemble Is a Set of Identical Experiments

The concept of an ensemble is basic to the study of probability. To discover the probability that a coin will land heads up, we flip a very large number of identical coins and count the fraction that lands heads up. Similarly, to discover the probability that a channel will be open under particular conditions, we could observe a large number of identical channels and count the fraction of open channels. This large number of identical experiments is called a statistical ensemble. To study a random system—one that will not give exactly the same result even when we start with exactly the same conditions—we must consider the average behavior of the system over many trials.

An ensemble can consist either of a set of identical experiments done on many identical systems or of a set of identical experiments performed serially on the same system. For a coin, we can either flip many coins or flip one coin many times. When we perform these experiments in reality, we need to worry about the condition that the experiments be identical. If we flip many coins, we need to know if the coins are really identical; if we flip one coin many times, we need to know if the coin changes with time or if it is affected by its past history. Similarly, if we study many channels, for instance, by measuring the average current in a patch with many channels or in a whole cell, we need to know if the channels really are identical. If we study a single channel over time, we need to know if the channel changes systematically with time and if its past history (many activating pulses) can affect its behavior (e.g., by slow inactivation). When we collect an ensemble of records of single-channel behavior, we study the same channel over a long time, so it is important to determine if the condition of long-term stability is met.

For a stationary process, such as a channel studied at a single voltage, no one time is inherently different from any other, so we can pool statistics of channel open and closed times regardless of where they occur in the record. However, for a nonstationary process, the lifetime of an opening or closing event can depend on the time it occurs. Thus, it makes no sense to average properties across different times in a record; rather, we must compare the behavior of many records at exactly the same time after the voltage step. This is the basis of ensemble statistics.

5. Ensemble Averaging Gives the Time-Dependent Probability of a Channel Being Open

Suppose we record from a patch containing a single Ca\(^{2+}\)- and voltage-activated potassium channel. We repetitively activate the channel with a depolarizing voltage step, taking care to wait between activating pulses so that the channel always starts from the same distribution of resting states. Figure 13-1 shows four traces from this kind of experiment. In order to find the probability of a channel being open at a time 2 msec after the step, we count the fraction of records in the ensemble that have
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a channel open at that time:

$$P_{\text{open}}(\text{at } t = 2 \text{ msec}) = \frac{\text{(number of records with channel open at } t = 2 \text{ msec)}}{\text{(total number of records in the ensemble)}}$$  (1)

If we perform this calculation at every time point, we get a picture of the channel-open probability as a function of time (see Fig. 13-1).

A practical approach to measuring channel-open probability, especially when more than one channel is in the patch, is to compute the ensemble mean. The ensemble mean number of channels open at time \( t \), \( \langle n(t) \rangle \), is given by

$$\langle n(t) \rangle = (1/M) \sum_{i=1}^{M} n_i(t)$$  (2)

where \( M \) is the number of records in the ensemble, and \( n_i(t) \) is the number of channels open at time \( t \) in the \( i \)th record. If the channels in the patch are independent, then the mean number of channels open is simply equal to the total number of channels present in the patch multiplied by the probability that a channel is open; thus, the probability of a channel being open at time \( t \) is given by

$$P_{\text{open}}(t) = \langle n(t) \rangle / N$$  (3)

where \( N \) is the total number of channels in the patch.

Although in some cases it is possible to see all of the channels open at once and directly count the number of channels present, this may be impossible for channels that inactivate. However, we can estimate the number of channels in the patch by calculating the ensemble variance. The ensemble variance is simply the mean square deviation of \( n(t) \):

$$\sigma^2(t) = \langle [n(t) - \langle n(t) \rangle]^2 \rangle = \langle n(t)^2 \rangle - \langle n(t) \rangle^2$$  (4)

For independent channels, binomial statistics gives:

$$N = \langle n(t) \rangle \cdot \left[ 1 - \sigma^2(t)/\langle n(t) \rangle \right]^{-1}$$  (5)

This relationship holds even for a large number of channels and has been used to estimate the number of channels present even when discrete channel-opening events cannot be resolved (Sigworth, 1977, 1980). The principle used here is the same as that for stationary fluctuation analysis, which is used to estimate the conductance and kinetics of channels in the steady state (Neher and Stevens, 1977). The essential difference between nonstationary and stationary fluctuation analysis is that in nonstationary fluctuation analysis each time point is considered separately.
6. Why Use Single-Channel Records?

Of course, the time-dependent probability derived from an ensemble average of a particular channel species resembles a record of the corresponding macroscopic current from a whole cell. The macroscopic current is given by

$$I = N \cdot i \cdot P_{\text{open}}(t)$$

(6)

where $N$ is the total number of channels and $i$ is the current through an open channel. So why should we go to the trouble of collecting many single-channel records, taking pains to make sure that they are all recorded under identical conditions, if we can get essentially the same information from a single record of macroscopic current through a large population of channels? An obvious advantage of single-channel records is that we can directly observe $N$ and $i$, so that we can get an appropriately normalized probability function. In addition, single-channel records give the $P_{\text{open}}(t)$ free from some of the artifacts of macroscopic recording. They also give additional kinds of experimental data, which can be used to test kinetic models, and they allow us to compute conditional averages to test specific hypotheses of channel behavior.

6.1. Single-Channel Recording Avoids Some Artifacts of Macroscopic Current Recording

One reason to prefer single-channel records to macroscopic current records is that some artifacts can make macroscopic currents look different from the channel-open probability, but they can be avoided in single-channel measurements. Four major problems with macroscopic currents are:

1. Current separation is imperfect. This means that some of the macroscopic current we measure is not going through the channels we are interested in. Even by manipulating ionic conditions and adding drugs to eliminate current through other channels, it is difficult to record current through only a single channel species.
2. Series resistance can make the true membrane potential different from the applied potential, and the true membrane potential will not be constant during a voltage step.
3. Ion accumulation can make the single-channel current change with time.
4. Voltage dependence of the open-channel conductance can be confused with voltage dependence of channel opening. To derive the probability of a channel being open from the macroscopic current, we need to know the size of the open-channel current at each potential of interest. This is usually inferred from the “instantaneous $I-V$,” a measurement that depends on our ability to measure current at a new potential very rapidly, before any channels open or close. Also, the instantaneous current is the sum of the open-channel current and the “leakage current” (all the conductances that
we are not interested in and could not eliminate), and we usually assume that
the leakage current is linear, which is rarely correct.

None of these artifacts is a serious problem in single-channel records, because we
can always measure the open-channel current for every opening event, and the
determination of the channel-open probability is independent of the measurement of
channel size. Since we can measure the open-channel size, we can distinguish
currents produced by different types of channels by their different sizes. Potential
changes caused by series resistance are negligible because of the small size of the
currents through a patch. Also, we can measure any change in channel size produced
by ion accumulation and correct for it. Of course, when we do ensemble analysis of
single-channel data, it often takes several minutes to acquire a sufficient amount of
data for a single good ensemble average; thus, experimental drift is a particular
concern in collecting ensembles of single-channel data.

Not only do single-channel records measure the channel-open probability
without the artifacts of macroscopic current measurements, but they also give
information about channel kinetics that is impossible to get from macroscopic
measurements. This advantage is most apparent with recordings from patches that
contain only a single channel. In this case, we know unambiguously the time and
duration of every opening event that contributed to the average.

6.2. Single-Channel Statistics Provide Further Bases for Testing Channel
Models

The viability of a kinetic model for channel function is tested by its ability to
reproduce experimental results. Traditionally, such a model must reproduce the
macroscopic currents recorded in voltage-clamp experiments. Similarly, for single-
channel data, a good model must predict the time course of the probability that a
channel is open (which parallels the time course of the macroscopic currents under
the conditions described above).

The added information we get from single-channel records, however, gives us
other functions that must be fitted by our kinetic model in addition to the time
course of the probability of a channel being open. It thus provides a more stringent
test of the model. We discuss briefly three such functions of channel behavior: the
open lifetime distribution, the closed lifetime distribution, and the first latency
distribution.

6.2.1. Open Lifetime Distribution

We can make a histogram of the fraction of open lifetimes less than a duration \( t \)
plotted against \( t \). As for stationary processes (see Chapter 9), this gives information
about the rate of closing.

Two features of the open lifetime distribution have interesting implications for
the molecular function of the channel: a nonexponential distribution of lifetimes and
a correlation between lifetime and time after the voltage step (latency). Channel
kinetics are usually described in terms of Markov processes. A lattice of states (which presumably correspond to conformational states of the channel molecule) is used to describe channel behavior, and the channel may jump from one state to another at various jump rates. We suppose the rates to be constant at a constant voltage; thus, the distribution of lifetimes of a single state is always exponential, with a characteristic lifetime determined by the reciprocal of the sum of the rate constants leaving that state. Three general variations of a simple model can give the complications mentioned above:

1. More than one open state. This kind of model will give an open lifetime distribution equal to the sum of several exponentials. Often such a model will also show a correlation between lifetime and latency, since the probability of entering one or the other open state may depend on the time after the pulse (latency). Obviously, we could directly distinguish different open states that have different conductances, but there may be several open states with indistinguishable conductances.

2. Time-dependent rate constants. A Markov process having rate constants that change with time is called time inhomogeneous. (This is different from nonstationarity, in which the probability of occupying various states changes with time, but the rate constants need not.) Physically, this might arise from a gradual relaxation of the channel molecule or of the membrane after the voltage step. Time-dependent rate constants will cause the lifetime distribution to be nonexponential, and openings occurring at different times will have different lifetimes.

3. Semi-Markov process. A semi-Markov process differs from a Markov process in that the distribution of lifetimes for a single state can be nonexponential (see Feller, 1964; Cox and Miller, 1965). In other words, the rate of leaving a state may vary with the time since the state was entered. Physically, this could result from a relaxation of the channel molecule or its environment after the channel opens. (This differs from time-inhomogeneous rate constants in that the "starting time" is the opening of the channel and not the voltage step.) We expect that such a process could show a nonexponential distribution of open lifetimes but not necessarily a correlation between lifetime and latency.

The standard approach to modeling channel kinetics has been to use Markov processes, and nonexponential distributions of open lifetimes have generally been explained in terms of multiple open states. However, we should not rule out the possibility that the physical mechanisms of channel function may be better represented by time-inhomogeneous or semi-Markov models.

6.2.2. Closed Lifetime Distribution

We can make a histogram of the fraction of closed lifetimes less than a duration $t$ plotted against $t$. Closed lifetime distributions give information about opening rates, and they can have all of the same complications discussed above for open lifetime distributions.
In compiling both open and closed lifetime distributions from records of repetitive voltage pulses, it is important to discard any event that is terminated by the end of the voltage pulse rather than by a subsequent gating transition, since it is impossible to know what the duration of the event would have been had the pulse not ended. However, this procedure can introduce a bias against observing long-duration events (see Fukushima, 1981, for a method of correcting for this bias).

6.2.3. First Latency Distributions

The time between the voltage step and the first channel opening is called the first latency. First latencies are treated in the theory of stochastic processes dealing with first passage times and recurrence times (Feller, 1950, 1966; Cox and Miller, 1965). A first passage time is the time at which a state is first entered, given an initial probability distribution among the states and a set of transition probabilities (rate constants). If the time is measured until the system reenters the initial state, the duration is called a recurrence time. For a single channel with one open state, the distribution of closed times is a recurrence time distribution.

Theoretical first latency distributions are calculated by finding the time course of the probability of being in the open state, with the open state treated as an absorbing state (a state with no exit). We are concerned only with the first time the open state is entered, and we need not consider what happens after that. The calculation of theoretical first latency distributions can be complicated, but it is possible for many simple channel-state models (see, for example, Fukushima, 1981; Patlak and Horn, 1982).

Each of these three distributions, in addition to the time-dependent probability of the channel being open, must be predicted by any model we make for the behavior of the channel at a molecular level. By providing this extra experimental information, single-channel data provide the basis for a more stringent test of channel models. Several investigators have taken advantage of this extra information in the analysis of Na⁺ channels (Fukushima, 1981; Horn et al., 1981; Patlak and Horn, 1982) and Ca²⁺ channels (Hagihara and Ohmori, 1983; Fenwick et al., 1982).

6.3. Multiple Channels in a Patch Reduce the Amount of Information Available

The presence of more than a single channel in a patch complicates the measurement of all three of the distribution functions discussed above. Open lifetime distributions with more than one channel present are confounded when channel openings overlap. Since it is impossible to tell which channel closed first, we cannot determine the lifetimes of these overlapping open events. Ignoring them leads to a bias against long openings. Similarly, closed time distributions are meaningless, because the closed intervals may be terminated either by the reopening of the channel that closed last or by the opening of another channel. Finally, the first latencies are shorter when multiple channels are present, since the latency observed is that of whichever channel opens first. If the number of channels is known, the
observed first latency distribution can be related to the distribution for a single channel (Patlak and Horn, 1982), but with too many channels, the observed latencies may be too short to measure accurately.

For small numbers of channels, these complications can be dealt with, but the amount of information in the data set is decreased. In order to make use of this information, however, we must know precisely how many channels are in the patch and must be sure that this number does not change during the course of the experiment.

6.4. Conditional Averaging Correlates Channel Behavior with Past or Future Channel Behavior

A powerful approach to analyzing ensembles of single-channel data is selective, or conditional, averaging. Members of the ensemble that satisfy a particular condition on the history of channel activity—for instance, those that have no channel open before t = 5 msec, those that have a channel open at t = 1 msec—are grouped together into a conditional average. The conditional average can be compared to the overall (unconditional) average of all the members of the ensemble or to other conditional averages to determine the effect of channel history on subsequent behavior of the channel. Since applications of the conditional averaging approach depend on the particular type of channels being studied, a specific example may best illustrate the approach. The following example is from a study of sodium channels in neuroblastoma cells by R. W. Aldrich and C. F. Stevens (1983).

Figure 13-2A shows an ensemble average of Na⁺ channel openings during a clamp step from −80 mV to +10 mV relative to the cell resting potential. This average gives the time course of the probability that a channel will be open. Figure 13-2B shows a similar ensemble average except that in this case, the test pulse to +10 mV is preceded by an 8-msec prepulse to −20 mV. The average shows that the probability of a channel being open during the test pulse is depressed compared to the test pulse without a prepulse. In addition, we see that a significant number of openings occurred during the prepulse. In terms of a state model for the Na⁺ channel, the probability of the channel opening during the test pulse is reduced because some of the channels inactivated during the prepulse—that is, they entered a long-lasting, nonconducting state. This experiment is equivalent to a macroscopic prepulse inactivation experiment.

Does the depolarizing prepulse cause inactivation only by causing channels to open and then inactivate, or is there a voltage-dependent inactivation of closed channels? In terms of kinetic schemes for the Na⁺ channel, we need to distinguish between these two possibilities:

Scheme I: \[ \begin{array}{c}
C \quad \xrightarrow{\text{O}} \\
\downarrow \quad \downarrow \\
1 \quad 1 
\end{array} \]

Scheme II: \[ \begin{array}{c}
C \quad \xrightarrow{\text{O}} \\
\downarrow \\
1 
\end{array} \]
Figure 13-2. Ensemble averages of single sodium-channel records from a cell-attached patch on a cultured mouse neuroblastoma cell. Leakage and capacitive currents were digitally subtracted from each record. Average A (N = 44) shows the time course of the probability of a channel being open \( P_{\text{open}}(t) \) during a 6-msec voltage pulse from \(-80\) to \(+10\) mV relative to the cell potential. Average B (N = 127) shows \( P_{\text{open}}(t) \) during an identical pulse that is preceded by an 8-msec prepulse to \(-20\) mV (relative to the cell potential). \( P_{\text{open}}(t) \) is depressed because of inactivation that occurs during the prepulse. The occurrence of openings during the prepulse can be seen in the average. This average can be decomposed into averages of records in which openings occur during the prepulse (C, N = 65) and those in which there were no prepulse openings (D, N = 62). The vertical axis (probability) is not calibrated because of uncertainty in the number of channels in the patch. This does not, however, affect the conclusion stated in the text. Note also that the calibration would be different during the prepulse and test pulse because of the different single-channel currents at these voltages, since the records were not scaled by the single-channel current, i. The temperature was 21°C.

where O indicates the open state of the channel and I represents an inactivated state; the boxed C represents a group of closed states. We want to know whether there is a pathway between some of the closed states and some inactivated (long-lasting closed) state. If there is, then the elevated voltage during the prepulse would cause some channels to inactivate even if they did not open, and the test pulse current would be reduced. If not, then channels must open during the prepulse in order to inactivate, and, on average, the test pulse current will only be reduced if channels actually opened during the prepulse.

If we look only at the average probabilities, or at the Na⁺ current in a macroscopic current experiment, we cannot distinguish these two general schemes for channel inactivation. But for each trace in our ensemble of single channel records, we know whether any channels opened during the prepulse. Suppose we separate all of our records into two categories, those that have an opening during the prepulse and those that do not. We can compute the average probability of opening during the test pulse for each category. These average probabilities (Fig. 13-2C, D) are called conditional averages: they show the probability that a channel is open given the condition that some channel did (or did not) open during the prepulse.
If scheme II is correct, then if no channels open during the prepulse, no channels can inactivate during the prepulse. Thus, the conditional probability of opening during the test pulse given that no channel opened during the prepulse (shown in Fig. 13-2D) should be the same as if there were no prepulse (the unconditional probability shown in Fig. 13-2A). However, if channels open during the prepulse, some may inactivate, and the probability of opening during the test pulse given that a channel opened during the prepulse (Fig. 13-2C) should be significantly reduced.

If scheme I is correct, however, then even if no channel opens during the prepulse, some channels may inactivate. In this case, the conditional probability of opening given that no channel opened during the prepulse (Fig. 13-2D) will indeed be reduced from the probability with no prepulse (Fig. 13-2A). Since, in this scheme, channels can inactivate from the closed or the open state, both conditional probabilities will be reduced compared to the probability of opening with no prepulse.

Figure 13-2 shows that, indeed, the two conditional probability averages are both smaller than the opening probability when no prepulse is used. This indicates that a significant amount of the inactivation during the prepulse resulted from inactivation directly from a resting closed state to an inactivated state without necessarily passing through the open state.

This simple example illustrates the power of using conditional averages to distinguish among various hypotheses of channel behavior. We were able to isolate a single aspect of the prepulse, channel opening, and study its relationship to channel inactivation. This question could be addressed directly, without constructing a complete model for the channel.

Analyzing the covariance of macroscopic currents also gives information about correlations between channel behavior at different times. This method involves computing the nonstationary autocovariance of the fluctuations in an ensemble of macroscopic current records (Sigworth, 1981). Because we know the exact history of channel behavior during each record in an ensemble of single-channel records, we can study historical correlations in channel behavior in a simpler and more direct way by using conditional averages.

7. Conclusion

Voltage-dependent channels are best studied by observing the relaxation of channel behavior after a voltage step. This is a nonstationary process, which can be studied by collecting an ensemble of single-channel records acquired under identical conditions. Ensembles of single-channel records can yield a picture of the time-dependent probability of a channel being open that is more reliable than that obtained from macroscopic current recording. Statistics of the individual opening and closing events, such as open and closed lifetimes and first latencies, provide additional empirical measures of channel behavior that must be predicted by any good kinetic model for the channel. In some cases, a specific kinetic model can be tested by computing conditional averages. Conditional averages correlate channel
behavior at different times. These approaches to studying single-channel data provide a powerful tool for elucidating channel kinetics.

References

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