Kinetic Time Constants Independent of Previous Single-Channel Activity Suggest Markov Gating for a Large Conductance Ca-activated K Channel

OWEN B. MCMANUS and KARL L. MAGLEBY

From the Department of Physiology and Biophysics R-430, University of Miami School of Medicine, Miami, Florida 33101

ABSTRACT Models for the gating of ion channels usually assume that the rate constants for leaving any given kinetic state are independent of previous channel activity. Although such discrete Markov models have been successful in describing channel gating, there is little direct evidence for the Markov assumption of time-invariant rate constants for constant conditions. This paper tests the Markov assumption by determining whether the single-channel kinetics of the large conductance Ca-activated K channel in cultured rat skeletal muscle are independent of previous single-channel activity. The experimental approach is to examine dwell-time distributions conditional on adjacent interval durations. The time constants of the exponential components describing the distributions are found to be independent of adjacent interval duration, and hence, previous channel activity. In contrast, the areas of the different components can change. Since the observed time constants are a function of the underlying rate constants for transitions among the kinetic states, the observation of time constants independent of previous channel activity suggests that the rate constants are also independent of previous channel activity. Thus, the channel kinetics are consistent with Markov gating. An observed dependent (inverse) relationship between durations of adjacent open and shut intervals together with Markov gating indicates that there are two or more independent transition pathways connecting open and shut states. Finally, no evidence is found to suggest that gating is not at thermodynamic equilibrium: the inverse relationship was independent of the time direction of analysis.

INTRODUCTION

Most studies of channel gating kinetics have assumed discrete states, with the transition rates among the states remaining constant in time for constant conditions (Col-

quhoun and Hawkes, 1977, 1981; Neher and Stevens, 1977; Hille, 1984; Horn, 1984; Bezanilla, 1985). Discrete states with transition rates independent of previous channel activity define a discrete Markov gating process. Although discrete Markov models have been useful in describing channel kinetics, there has been little direct evidence for the assumption that the transition rates are independent of previous channel activity. If this Markov assumption for channel gating were found to be in error, then a reinterpretation of most of the previously considered gating mechanisms would be necessary. Some support for Markov gating has been obtained. Magleby and Stevens (1972) found that end-plate currents decay with a time course independent of the previous holding potential, and hence channel activity. Hahin (1988) has made a similar observation for the decay of sodium tail currents after removal of inactivation. Pallotta (1985a) has observed that the lifetime of a brief open state for a large conductance Ca-activated K channel (BK channel; Marty, 1981; Pallotta et al., 1981; Latorre et al., 1982) is independent of inactivation, and hence, previous channel activity. Colquhoun and Sakmann (1985) have found for acetylcholine receptor channels that there is no asymmetry in bursts of openings, and Horn and Vandenberg (1984) and Keller et al. (1986) have found exponential open dwell times for single Na channels, consistent with Markov gating.

The purpose of our study is to extend the investigation of the Markov assumption using detailed single-channel analysis. We examine whether the gating of the BK channel is consistent with the basic Markov assumption of rate constants independent of previous channel activity. We also investigate if the gating is consistent with thermodynamic equilibrium (microscopic reversibility), and we investigate the transition pathways among the states. The experimental approach is to analyze the durations of adjacent open and shut intervals in current records obtained from excised patches of rat muscle membrane containing single active BK channels. If the gating kinetics of the channel are described by discrete states with constant transition rates, then the time constants of the exponential components in conditional distributions of interval durations should be independent of the durations of the adjacent intervals used to select the conditional intervals (Fredkin et al., 1985).

It is found that the time constants in the conditional distributions are independent of the durations of adjacent interval durations. This observation is consistent with the Markov assumption that transition rates are independent of previous channel activity. It is also found that the inverse relationship between the mean durations of adjacent open and closed intervals (McManus et al., 1985) is independent of the time direction of analysis. Violation of time reversal in the data would indicate that channel gating is not in thermodynamic equilibrium (Colquhoun and Hawkes, 1983; Läuger, 1983; Colquhoun and Sakmann, 1985; Steinberg, 1987; Kerry et al., 1988). Finally, the combined observation of Markov gating and a dependent relationship between the durations of adjacent open and shut intervals indicates two or more independent transition pathways connecting open and shut states. The data also suggest that the effective lifetimes of the open states are, in general, inversely related to the effective lifetimes of the shut states to which they make direct transitions. An abstract of some of the results has appeared (Magleby and McManus, 1985).

METHODS

Excised Patches Containing a Single Active BK Channel

Currents flowing through single large conductance calcium-activated potassium channels (BK channels, Marty, 1981) in surface membrane of primary cultures of rat skeletal muscle (myotubes) were recorded with the patch-clamp technique (Hamill et al., 1981). Myotubes were prepared as described previously (Barrett et al., 1982). All experiments were performed with excised inside-out patches of membrane containing a single active BK channel. Single-channel patches were identified by observing current steps to only a single level under extended observations at high open probability. Membrane potentials for the excised patches are reported in the same manner typically used for intact cells, as the voltage at the normal intracellular side of the membrane with respect to the extracellular side. Experiments were performed at room temperature (22–24°C). Some of the methods are the same as those described in greater detail previously (McManus and Magleby, 1988).

Solutions

The solutions bathing both sides of the membrane contained (in millimolar): 144 KCl, 2 TES buffer (N-tris [hydroxymethyl-2-aminoethane sulfonic acid]), 1 EGTA (ethyleneglycol-bis-[b-aminoethyl ether]N,N'-tetra-acetic acid). The solution in the patch pipette bathing the extracellular membrane surface contained no added Ca^{2+} giving a free Ca^{2+} of $<10^{-9}$ M. Ca^{2+} was added to the solutions used to bathe the inner membrane surface to activate the channel. The free Ca^{2+} concentrations in the solutions at the inner membrane surface are indicated in the paper for the various experiments and were determined with the method described by Bers (1982) which uses a Ca^{2+} sensitive electrode. A detailed description of our application of this method is in McManus and Magleby (1988).

Solutions were adjusted to either pH 7.0 or 7.2, as indicated. Although the 0.2 pH unit difference did appear to have some effects on channel activity, these effects are not dealt with in this paper, as the conclusions reached from the adjacent interval analysis were the same for the two slightly different pH values.

Recording and Filtering to Exclude False Events

Single-channel currents were recorded and stored on FM tape (frequency response, DC to 20 or 40 kHz, −3 dB) during experiments. Data on tape were then actively low-pass filtered at 24 dB/octave (model 4302 in pulse mode; Ithaco, Inc., Ithaca, NY) to give a final effective cut-off frequency, including the patch clamp, tape recorder, and filter, of 6–8 kHz (−3 dB). Effective filtering was measured in terms of dead time, determined by the duration of a rectangular pulse, which results in the filtered pulse reaching 50% of its true amplitude (Colquhoun and Sigworth, 1983). Dead times ranged from 20 to 50 μs, with most of the experiments having dead times of 25–30 μs.

Detection of even a limited number of noise peaks as (false) channel events would result in incorrect results for the types of analysis carried out in this paper. Consequently, the filtering was set so that no noise peaks should have exceeded the 50% threshold level for detection of channel opening or closing. This was done by adjusting the filtering so that noise in the direction opposite to channel opening, which is not contaminated by channel events, did not exceed 50% of the absolute single-channel amplitude (details in McManus et al., 1987). With sufficient filtering to eliminate detection of noise peaks as false events, the standard deviation of the baseline noise in the absence of channel activity was typically ~7–9% of the single-channel amplitude.

Measuring Interval Durations

The recorded and filtered single-channel currents were played into an 11/73 computer (Digital Equipment Corp., Maynard, MA) at one-eighth to one-sixteenth normal tape speed for on-line measuring of open and shut interval durations. Interval durations were measured by 50% threshold detection (Colquhoun and Sigworth, 1983) and saved sequentially in files for later analysis. Such automated analysis provided the necessary means to measure the approximately two million intervals analyzed for this study.

As pointed out by Colquhoun (1988), automated analysis of single-channel events can give erroneous results if improperly applied. To avoid potential errors associated with automated analysis several precautions were taken.

(a) The data were always examined on an oscilloscope as it was played into the computer at greatly reduced speed, which allowed noise artifacts, such as can occur from defects in the tape, to be identified and excluded from analysis.

(b) Only data with stable baselines were analyzed. For many experiments, the baseline drift was less than a few percent of the single-channel amplitudes and could be analyzed without baseline adjustment. In experiments where baseline drift exceeded this, the reduced tape speed allowed sufficient time for visual adjustment of the baseline, as necessary, during analysis. Tests indicated that such visual adjustment could control the baseline drift to $< \pm 5\%$ of the single-channel amplitude.

(c) The effective sampling rates of 5–15 μs were sufficiently fast to reduce sampling detection and sampling promotion errors resulting from discrete sampling to negligible levels (McManus et al., 1987).

(d) Under our experimental conditions, $\sim 0.1\%$ of the openings for the BK channel are to subconductance states (Barrett et al., 1982). Such subconductance states can generate bursts of very brief open and shut intervals with 50% threshold analysis (Colquhoun, 1988). Erroneous bursts of detected intervals due to subconductance states were identified in stability plots of mean open and shut interval durations, averaged 5–10 at a time, (McManus and Magleby, 1988), and excluded from analysis. Such erroneous bursts arising from subconductance states were easily identified, as they gave brief pronounced drops in the mean open and shut intervals on the stability plots that exceeded any drops in the mean interval durations due to normal channel kinetics. The magnitude of drops in the mean associated with subconductance states and normal channel kinetics were established for each experiment by comparing the stability plots with visual inspection of the single-channel current record.

Although erroneous bursts from subconductance states were identified and excluded, together with several of the preceding and following intervals, doing so had little effect on the time constants in the distributions of interval durations. This was the case because the subconductance states occurred infrequently, and the erroneous intervals arising from such states were typically less than two times the dead time in duration, so they were not included in the fitting (see below). In contrast, erroneous data from subconductance states, if they were not excluded, would have affected the plots of adjacent mean interval durations, as these plots included data with very brief intervals.

Determining the Relation between the Mean Durations of Adjacent Open and Shut Intervals

The files containing the durations of sequential open and shut intervals during normal channel activity (see McManus and Magleby, 1988) were scanned, and the open intervals were separated into groups depending on the duration of the adjacent shut intervals. When the sequence in the intervals was interrupted due to the exclusion of erroneous intervals, such as those associated with subconductance states, the scan was stopped and then restarted to avoid

improper juxtaposition of intervals. Open intervals occurring immediately before and immediately after shut intervals of specified durations were tabulated separately. The mean durations of the preceding open intervals, the following open intervals, and both combined were then plotted against the mean duration of the shut intervals for each specified range of shut interval durations. Such adjacent interval plots indicate the dependence of the mean open time on the duration of the adjacent shut intervals. The dependence of the mean shut time on the duration of the adjacent open intervals was calculated in the same way, after sorting the shut times into groups based on the durations of adjacent open intervals.

Conditional Open and Shut Distributions

Conditional open-time distributions were constructed from the open intervals occurring before and after shut intervals of specified durations. Conditional shut-time distributions were similarly constructed based on the adjacent open interval durations. Because large numbers of events are required to define exponential components, only two or three conditional distributions were constructed from each experimental data set. The conditional distributions were then binned, plotted, and fitted in the same manner as the (unconditional) distributions of all open or shut intervals (see below).

Binning and Plotting Interval Durations

Open and closed intervals were binned according to the logarithms of their durations, as described in McManus et al. (1987). Log binning allows intervals of any observed duration to be binned in a few hundred bins with negligible error if the resolution is 25 bins or greater per log unit. Larger bins can also be used without error if a correction for bin width is applied during the fitting (McManus et al., 1987).

For plotting, the number of plotted intervals in each bin N, $N_{plot}(N)$, were scaled for bin width with

$$N_{plot}(N) = N_{obs}(N)\ B(1)/B(N), \tag{1}$$

where $B(1)$ and $B(N)$ are the bin durations (widths) for the first bin and bin N, respectively, and $N_{obs}(N)$ is the number of intervals in bin N. With this correction for bin width, the plotted histogram has the same form as the probability density function fit to the data. If the log bins are not divided by bin width, then plots of the form described by Sigworth and Sine (1987) are obtained.

Fitting Distributions of Interval Durations with Sums of Exponentials

Distributions of open and shut interval durations were fit with sums of exponential components using the method of maximum likelihood (Colquhoun and Sigworth, 1983), as applied to binned and sampled data (McManus et al., 1987). Each distribution was fit with

$$f(t) = \sum_{j=1}^{k} a_j\, \tau_j^{-1}\, e^{-t/\tau_j} \tag{2}$$

where $f(t)$ is the probability density function (PDF) for the sum of k exponential components, and τ_j and a_j are the time constant and area, respectively, of component j. The durations of all intervals greater than twice the dead time were fit by maximizing the likelihood of the parameters (areas and time constants) describing the exponential components. One of the areas is not a free parameter, as the areas sum to one in a PDF. Intervals less than two times the dead time were excluded to prevent detection of possible "phantom" exponentials, which can occur when data are filtered (Roux and Sauve, 1985; Blatz and Magleby, 1986a) and because

durations of filtered intervals less than two dead times are underestimated with 50% detection (Colquhoun and Sigworth, 1983). Correction for such reduced durations is impractical because of the complications introduced by the noise in the experimental record (McManus et al., 1987). For visual comparison of the fitted PDF to the data, the PDF was scaled by multiplying $f(t)$ by the bin width of the first plotted bin and by the estimated number of events in the fitted distribution, calculated with Eq. 18 in McManus et al. (1987).

Determining the Number of Exponential Components

The number of significant exponential components in the distributions of interval durations was determined with the likelihood ratio test (Rao, 1973; Horn and Lange, 1983). Each distribution was fitted with increasing numbers of summed exponential components and the fits with different numbers of exponentials were compared by determining $\log_e R_k$ from

$$\log_e R_k = \log_e (L_k/L_{k-1}) = \log_e L_k - \log_e L_{k-1} \tag{3}$$

where $\log_e R_k$ is the natural logarithm of the likelihood ratio for k vs. $k - 1$ exponential components, and L_k and L_{k-1} are the maximum likelihoods that the data were drawn from a distribution described by the sum of k and $k - 1$ exponential components. An additional exponential component was judged significant ($P < 0.05$) when twice the value of $\log_e R_k$ was >5.99, the χ^2 value for two degrees of freedom at the 0.05 level. The criteria of 0.05 was selected based on analysis of simulated data with distributions similar to those of the experimental data and from the analysis of experimental data sets divided into smaller ones so that the effect of numbers of events on detection of components could be determined (see McManus and Magleby, 1988). Such analysis indicated that, for the sample sizes used, a more stringent criteria could exclude the detection of some exponential components likely to be present in the data.

Using Resampling to Determine If the Ratios of the Means of the Preceding to Following Intervals Are Significantly Different from One

Resampling methods (Efron, 1982; Horn, 1987) were used to determine whether the differences between preceding and following intervals shown in Fig. 2 might occur by chance alone. For each specified range of intervals, the original set of preceding and of following adjacent interval durations was resampled with replacement to obtain an artificial data set with the same number of pairs of preceding and following intervals as in the original data set. Each artificial distribution was generated by drawing individual pairs of preceding and following intervals at random from the original data set, with the stipulation that each pair drawn from the original was replaced. Thus, for each artificial data set, some of the pairs in the original data set may appear more than once and some of the pairs may not appear at all. The ratio of the mean duration of the preceding intervals to the mean duration of the following intervals was then calculated from the artificial data set of resampled pairs. This entire procedure was then repeated 200 times to obtain the expected distribution of the ratio of the means (of the preceding to following adjacent interval durations) that might occur due to chance alone for the original data set.

If $>95\%$ of the ratios obtained from the 200 resampled artificial data sets were greater than one for an original data set with a ratio greater than one, or less than one for an original data set with a ratio less than one, then the ratio of the original data set was considered to be significantly different from one ($P \leq 0.05$). Testing for significance directly from the distribution of ratios in this manner made the significance test independent of the form of the distribution of ratios.

Using Resampling to Estimate Errors in Magnitudes and Time Constants

Resampling methods were also used to obtain estimates of errors associated with determining the magnitudes and time constants of the exponential components fitted to the distributions. Typically, 20 artificial distributions were generated from each original distribution by resampling with replacement. The artificial distributions, all of which contained the same number of events as the original distribution, were then fit with sums of exponentials using the same methods as those used for the original distribution. The standard deviation of the fitted magnitudes and time constants in the 20 artificial distributions then gave an estimate of the expected variability in determining the exponential parameters from the original distributions. The estimated standard deviations are plotted as error bars in Figs. 4 and 7 (see below). In many cases the error bars are less than the symbol size, and hence are not visible.

Estimating the Dispersion of the Median for the Change in Conditional Distributions

The above section used resampling to estimate errors associated with determining the exponential components describing dwell-time distributions from a single experiment. Estimates of errors were also determined by analyzing data from several experiments. In comparing data from several experiments, the question of interest was whether the areas and time constants of the different exponential components changed in a consistent manner in the different experiments, rather than the absolute values of the parameters, which could vary among experiments because of differences in open probability. To estimate errors associated with the change of the conditional distributions as a function of the durations of the adjacent intervals, the areas and time constants of the exponential components in the conditional distributions for each experiment were normalized by expressing them as a ratio of the areas and time constants of the components in the unconditional distributions in the same experiment.

The medians and dispersion of the medians (see below), in terms of ratios, for all the experiments were then determined from the normalized data. For plotting the data, the median and dispersion of the median of the ratios for each parameter were expressed in terms of the mean of the conditional parameters for all the experiments. Normalizing and plotting the data in this manner allowed a visual assessment of the change in the conditional parameters as a function of adjacent interval duration to be determined, rather than differences among experiments.

When data are normalized by dividing the experimental (conditional) parameters by the control (unconditional) value, the resulting distributions of ratios are skewed due to the asymmetry resulting from the division, even if the distributions of the experimental and control parameters are normal (Colquhoun, 1971). Numerical analysis of distributions of ratios formed from values drawn from normal distributions indicated that the deviation from normal would be small when the standard deviations of the normal distributions were <5% of the mean values. The skew became considerable when the standard deviation exceeded 20% of the mean, and if values from the control distribution approached zero (not likely to happen for the experimental data), then the skew could approach infinity, because of division by small numbers.

To investigate the effects of the skew on the normalized data, the ratios were analyzed in two ways. First, the medians and bidirectional dispersion of medians (details below) were determined and plotted, as in Figs. 5 and 8. Second, the means and standard errors of the means were calculated and plotted (not shown). The results of the two types of analysis (means and medians) were almost identical on the log-log plots, and the lengths of the errors bars were similar. Hence, any effects of the skew would be minimal since the medians (which

exclude the values of the extreme data points) and the means (which include the values of all data points) were almost identical.

Statistical tests for changes in the areas and time constants of the conditional distributions were made in two ways: (a) from the original data sets before normalization, to exclude any affects of the skew of the ratios, and (b) because the effect of the skew in the distributions of ratios was minimal, from the ratios, which then allowed the change in the parameters to be assessed, independent of differences in absolute values among the experiments. Changes in parameters were considered significant for P values ≤ 0.05 for paired t tests of conditional and unconditional values.

The median and dispersion of median for the points in Figs. 5 and 8 were calculated as follows: the median data point, m, of n ranked data points was given by $(n + 1)/2$. The median value was then the value (or interpolated value for data sets with an even number of observations) of the median data point. The plotted error bars include the middle two quarters of the data points, assuming a continuous distribution. Defining q as $m/4$, the lower limit at $1/4$ was obtained from the interpolated value at $q + 1$ data points, and the upper limit at $3/4$ was obtained from the interpolated value at $n - q$ data points (van der Waerden, 1957).
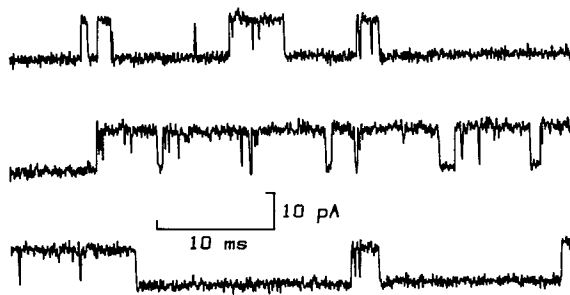

FIGURE 1. Currents through a single large conductance Ca-activated K channel in an excised membrane patch from cultured rat skeletal muscle. Upward current steps indicate channel opening. The three traces are continuous in time. Effective low-pass filtering (24 dB/octave, -3 dB) of 5.87 kHz to give a dead time of 30 μs. P_o = 0.70; membrane potential = $+30$ mV; Ca_i = 23 μM; pH 7.0.

RESULTS

Currents during Normal Activity

The large conductance Ca-activated K channel (BK channel) can enter at least four different kinetic modes, with ~96% of the intervals occurring during activity in the normal mode (McManus and Magleby, 1988). The results presented in this paper are for normal mode activity, as identified by stability plots of mean open and shut interval duration (McManus and Magleby, 1988). To simplify the interpretation of the data, only membrane patches containing single BK channels were analyzed. Currents through a single BK channel during normal activity are shown in Fig. 1, in which upward current steps indicate opening. The gating kinetics appear complex, as might be expected for transitions among three to four open and six to eight shut kinetic states during normal activity (McManus and Magleby, 1988).

Mean Durations of Open and Shut Intervals Are Inversely Related

From the records in Fig. 1 it appears that the briefer openings often occur adjacent to the longer shut intervals, whereas the longer openings often occur adjacent to the briefer shut intervals. Fig. 2, which plots the relationship between the durations of adjacent open and shut intervals, shows that this is the case. Sequential open and shut intervals were first scanned to find all shut intervals with durations falling within eight specified ranges (see figure legend). The mean durations of all open
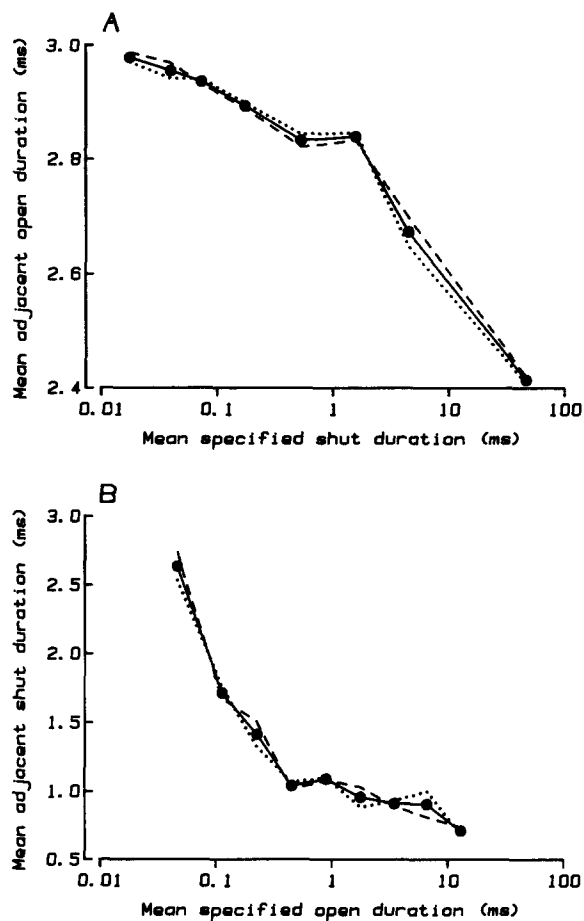

FIGURE 2. Relationship between the mean durations of adjacent open and shut intervals. (A) A file of 233,226 sequential intervals was scanned to sort the shut intervals into eight specified ranges of durations (in milliseconds): 0–0.025, 0.026–0.052, 0.053–0.102, 0.103–0.302, 0.303–0.925, 0.926–2.702, 2.703–8.102, and >8.102. The mean durations of the open intervals adjacent to (both before and after) the shut intervals in each specified range are plotted (*filled circles*) against the mean durations of the shut intervals in each specified range. The mean durations of adjacent open intervals immediately before (*dotted line*) and after (*dashed line*) the shut intervals in each specified range are also plotted. The lines have experimental values only at the times of the plotted points. (B) Open intervals from the same data file were sorted into nine specified ranges of durations (in milliseconds): 0–0.077, 0.078–0.152, 0.153–0.302, 0.303–0.602, 0.603–1.202, 1.203–2.402, 2.403–4.802, 4.803–9.602, and >9.602. The mean durations of the shut intervals adjacent to the open intervals in each specified range are plotted (*filled circles*) against the mean durations of the open intervals in each specified range. The mean durations of adjacent shut intervals immediately before (*dotted line*) and after (*dashed line*) the shut intervals in each specified range are also plotted. Shut times greater than 1,000 ms were set to 1,000 ms in B to reduce variability due to a few very long shut times. Same experiment as Fig. 1.

intervals adjacent to (immediately preceding and following) the shut intervals in each specified range were then determined. The filled symbols connected by the continuous line plot the mean duration of the adjacent open intervals, both immediately before and after the specified shut intervals, against the mean of the shut intervals in each range. The dotted line connects the mean durations of only those adjacent open intervals immediately preceding the specified shut intervals, and the dashed line connects the mean durations of only those adjacent open intervals immediately following the specified shut intervals. The lines have experimentally determined values only at the times of the filled symbols.

Fig. 2 A shows that, except for the slight peak at 1–2 ms, the longer the duration of the specified shut interval, the briefer the mean duration of the adjacent open intervals; this was the case for open intervals both before and after the specified shut intervals.

Fig. 2 B plots the mean durations of shut intervals adjacent to open intervals of specified durations (continuous line). The mean shut times before (dotted line) and after (dashed line) the specified open intervals are also plotted. It can be seen that increasing the durations of the specified open times led to briefer mean durations of the adjacent shut intervals. The data in Fig. 2 B were analyzed by setting all shut intervals longer than 1,000 ms to 1,000 ms. This was done in order to reduce the large amount of variability that can arise from the very long shut intervals that contribute <1% of the shut events. Simulations showed that truncating the durations of long intervals in this manner preserved information about the durations of adjacent intervals while giving an estimate of the mean, which was not likely to be worse than estimates obtained without truncation, and which was typically better. (See also the discussion of trimmed means in Lehmann, 1983). Similar results, but with more variability, were obtained when the long shut intervals were not set to 1,000 ms.

A similar inverse relationship between the durations of adjacent open and shut intervals was observed in 18 additional data sets of normal activity from eight different BK channels for open probabilities (P_o) ranging from <0.01 to 0.7, depending on the Ca_i. In general, the magnitude of the inverse relationship became more pronounced than in Fig. 2 when P_o was decreased by decreasing Ca_i, as long as P_o was greater than ~0.05. (P_o was 0.7 for Figs. 1 and 2.)

A plateau or slight peak near 1–2 ms, which could be more pronounced than is evident in Fig. 2 A, was typically seen in plots of this type when the P_o was >0.05.

Testing for Thermodynamic Equilibrium

The single-channel currents presented in Fig. 1 and analyzed for the plots presented in Fig. 2 were recorded under nonequilibrium conditions, since a potential difference across the membrane of 30 mV was used to generate a net flux of ions through the open channel. The flux of ions could provide energy to alter channel gating so that the various states of the channel may not be in thermodynamic equilibrium (Colquhoun and Hawkes, 1983; Läuger, 1983; Colquhoun and Sakmann, 1985; Steinberg, 1987), even though channel activity is in a steady state, as indicated by stability plots. The data in Fig. 2 provide a means to investigate this possibility.

If a channel is in thermodynamic equilibrium so that microscopic reversibility is maintained, then the relationships between various intervals should be the same

independent of whether the data are analyzed forward or backward in time (Colquhoun and Hawkes, 1983; Läuger, 1983; 1987; Colquhoun and Sakmann, 1985; Steinberg, 1987; Ball et al., 1988). On this basis, the relationship between the mean durations of adjacent intervals should be independent of whether an interval occurs immediately before or immediately after an interval of specified duration (Kerry et al., 1988).

In Fig. 2, the mean durations of the intervals immediately before (*dotted lines*) and immediately after (*dashed lines*) intervals of specified durations were similar to the overall mean (*filled circles*), and this was the case independent of whether open intervals adjacent to specified shut intervals were examined (Fig. 2 *A*) or whether shut intervals adjacent to specified open intervals were examined (Fig. 2 *B*). Although there was an absence of a general effect of time direction on adjacent interval durations, there were some small differences for some of the points.

To investigate whether the small differences between preceding and following intervals shown in Fig. 2 might occur by chance alone, resampling techniques (Efron, 1982; Horn, 1987) were used to estimate the expected variability (details in Methods). For 16 of the 17 specified durations in Fig. 2, *A* and *B*, the ratios of the means of the preceding to the following intervals were not significantly different from 1.0. The one specified duration in which the ratio was significantly different from 1.0 occurred for the next to last specified range (4.8–9.6 ms) in Fig. 2 *B*.

Such an observation, considered separately, would suggest deviation from microscopic reversibility. This may not be the case, however, as 1 of 20 tested ratios would be expected to be significantly different from 1.0 at the 0.05 level just due to chance alone, even if the underlying process generated preceding and following intervals of equal mean durations. Furthermore, when comparisons were made between the means of the preceding and following intervals in 14 different data sets, no consistent pattern or direction was seen in any deviations of the ratios from 1.0 (not shown).
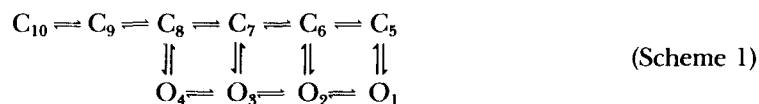
Thus, we have found no consistent evidence to support a deviation from thermodynamic equilibrium. A similar finding was made for the glutamate receptor channel by Kerry et al. (1988). The absence of such evidence does not, however, necessarily establish that the channel is at thermodynamic equilibrium (see Discussion).

In any case, the data in Fig. 2 do show that there is a general inverse relationship between the durations of adjacent open and shut intervals (McManus et al., 1985), and that this inverse relationship is independent of the time direction of analysis. The observation that the inverse relationship is independent of the time direction allows those adjacent intervals both before and after those intervals of specified durations to be combined, increasing the number of events available for the detailed analysis to follow.
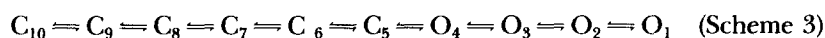
Interpretation of the Inverse Relationship Can Differ for Some Markov and Non-Markov Gating Processes

This section considers two fundamentally different models that can generate an inverse relationship between the mean durations of adjacent open and shut intervals. The following sections then outline a method to distinguish between these gating mechanisms and to apply the method to single-channel data.

The first gating mechanism to be considered assumes that gating follows a discrete Markov process, such that the mean dwell time in each state is independent of the previously entered states or their dwell times. For a discrete Markov model, an inverse relationship could be generated if there were two or more independent transition pathways (or gateway states) between the open and shut states and if the briefer duration open states made direct connections to the longer duration shut states (McManus et al., 1985). For example, consider the general gating mechanism described by scheme 1. If the mean lifetimes of the shut states decrease from C_{10} through C_5, and the mean lifetimes of the open states increase from states O_4 to O_1, then longer open intervals would, on average, be more likely to occur adjacent to briefer shut intervals, as observed in Fig. 2. (The slight peak at 1–2 ms in Fig. 2 A suggests that the relationship between states is probably not as systematic as this.)

$$C_{10} \rightleftharpoons C_9 \rightleftharpoons C_8 \rightleftharpoons C_7 \rightleftharpoons C_6 \rightleftharpoons C_5$$
$$\updownarrow \quad \updownarrow \quad \updownarrow \quad \updownarrow \qquad \text{(Scheme 1)}$$
$$O_4 \rightleftharpoons O_3 \rightleftharpoons O_2 \rightleftharpoons O_1$$

The second general gating mechanism to be considered assumes that gating does not follow a discrete Markov process. On this basis the inverse relationship between the durations of adjacent open and shut intervals can arise from models with a single transition pathway between the open and shut states, such as schemes 2 and 3, if the rate constants are dependent on the previous history of channel activity (non-Markov process). For example, if the rate constants for closing from O_1 to C_2 in scheme 2, or from O_4 to C_5 in scheme 3, were directly related to the dwell time spent in the previous shut state or states, then an inverse relationship between adjacent open and shut intervals could occur.

$$C_2 \rightleftharpoons O_1 \qquad \text{(Scheme 2)}$$

$$C_{10} \rightleftharpoons C_9 \rightleftharpoons C_8 \rightleftharpoons C_7 \rightleftharpoons C_6 \rightleftharpoons C_5 \rightleftharpoons O_4 \rightleftharpoons O_3 \rightleftharpoons O_2 \rightleftharpoons O_1 \quad \text{(Scheme 3)}$$

It should be noted that schemes with a single gateway state, such as schemes 2 and 3, have previously been rejected for the BK channel (McManus et al., 1985), but this rejection was based on the untested assumption of Markov gating. For Markov gating, schemes with a single transition pathway between open and shut intervals predict an independent relationship between the durations of adjacent interval durations (Fredkin et al., 1985; McManus et al., 1985; Blatz and Magleby, 1989) in contrast to the observed dependent relationship in Fig. 2. However, if gating is non-Markovian so that the rate constants can change in a dependent manner with previous channel activity, then schemes with one gateway state cannot be rejected on the basis of the data in Fig. 2, as such schemes can generate dependent relationships between adjacent interval durations.

Testing for Markov Gating

For scheme 1 with Markov gating, the inverse relationship between adjacent open and shut dwell times arises because a series of open and shut states with inversely related (effective) dwell times are connected by multiple transition pathways; the rate constants, and hence the lifetimes of the various states, are fixed, being inde-

pendent of the previous history of channel activity. In contrast, for schemes 2 and 3 with non-Markov gating, the inverse relationship arises because the rate constants, and hence dwell times, change as a function of previous activity. These specific Markov and non-Markov mechanisms for channel gating would be distinguishable, then, if it could be determined whether the rate constants remain fixed or change with previous channel activity.

One method of investigating whether the rate constants are dependent on previous channel activity is to examine distributions of interval durations, conditional on the durations of preceding intervals (Fredkin et al., 1985). Preceding intervals of different durations would indicate differences in channel activity, and any changes in the rate constants associated with these differences in channel activity would be reflected in the conditional distributions of following intervals. For Markov gating, the time constants of the exponential components in the conditional distributions should be independent of the durations of the specified preceding intervals used to select the conditional distributions, whereas the areas of the exponential components would be dependent (Fredkin et al., 1985). This can be shown as follows. The unconditional distributions of all open $f_o(t_o)$ and all closed $f_c(t_c)$ dwell times are described by the sums of exponential components, with one component for each state:

$$f_c(t_c) = \sum_{i=1}^{N_c} \alpha_i \, e^{-(t_c/\tau_i)} \tag{4}$$

$$f_o(t_o) = \sum_{j=1}^{N_o} \beta_j \, e^{-(t_o/\tau_j)} \tag{5}$$

where N_c and N_o are the number of closed and open states, α_i and β_j are the magnitudes, and τ_i and τ_j are the time constants of the components of the closed and open distributions, respectively (e.g., Colquhoun and Hawkes, 1983). The two-dimensional distribution (which gives a three-dimensional surface) of an open dwell time and the following closed dwell time, $f_c(t_o, t_c)$, is given by (Fredkin et al., 1985, from Eq. 4.1):

$$f_{oc}(t_o, t_c) = \sum_{i=1}^{N_c} \sum_{j=1}^{N_o} \nu_{ij} \, e^{-(t_o/\tau_j)-(t_c/\tau_i)} \tag{6}$$

Eq. 6 indicates that the observed two-dimensional distribution of all pairs of open-shut dwell times for a Markov model with N_c shut and N_o open states would have N_c shut and N_o open exponential components. The observed distribution would be formed by the sum of $(N_c \times N_o)$ two-dimensional distributions, each with one open and one shut exponential component with identical magnitudes, ν_{ij}. For example, for a model with four open and six shut states, 24 two-dimensional distributions, each with one open and one shut exponential component, would sum to form the observed two-dimensional distribution.

Eq. 6 can be rearranged to:

$$f_{oc}(t_o, t_c) = \sum_{i=1}^{N_c} \left[\sum_{j=1}^{N_o} \nu_{ij} \, e^{-(t_o/\tau_j)} \right] e^{-(t_c/\tau_i)} \tag{7}$$

$$= \sum_{i=1}^{N_c} \delta_i \, e^{\,-(t_c/\tau_i)} \tag{8}$$

where

$$\delta_i = \sum_{j=1}^{N_o} \nu_{ij} \, e^{-(t_o/\tau_j)}. \tag{9}$$

Eq. 8 shows that for any given open dwell time, t_o, the conditional distribution of closed dwell times after the given open time would have the same time constants, τ_i, as the unconditional distribution of all closed dwell times (Eq. 4). In contrast, the magnitudes, δ_i, and consequently, also the areas, of the closed exponential components would be dependent on the open time (Eq. 9). Thus, for Markov gating, the time constants of the observed exponential components in the conditional shut distribution should be independent of the durations of the specified adjacent open intervals used to select the conditional shut intervals, whereas the areas should be dependent. Similar equations and conclusions apply to conditional open times adjacent to specified shut times. Since the observed time constants are determined by the rate constants (e.g., Colquhoun and Hawkes, 1983), an observation of time constants independent of previous channel activity would suggest rate constants independent of previous channel activity, and Markov gating. (Additional tests for Markov gating are presented in Fredkin et al., 1985.)

Missed Events and Testing for Markov Gating

Eqs. 4–9 and the conclusion of time constants independent of previous channel activity for Markov gating are for pairs of intervals with unlimited time resolution. Consequently, these equations will not be exact for experimental data with limited time resolution and missed events. This is the case since missed events can prolong the time constants of some of the expected exponential components in the dwell-time distributions and add additional components at brief times (Colquhoun and Sigworth, 1983; Roux and Sauve, 1985; Blatz and Magleby, 1986a; Ball and Sansom, 1988; Yeo et al., 1988).

To examine whether an observation of time constants independent of previous channel activity would still be consistent with Markov gating for experimental data with limited time resolution and for conditional distributions of the type analyzed in the following sections where marginal distributions may also contribute, simulated data were analyzed. In these tests 5×10^5–10^6 intervals were simulated with limited time resolution (simulation details in Blatz and Magleby, 1986a) using scheme 1 and other schemes previously described for the gating of the BK channel (Magleby and Pallotta, 1983a; McManus and Magleby, 1986). The simulated intervals were then binned into unconditional and conditional distributions in the same manner used in the following sections for the experimental data, and the areas and time constants of the fitted exponential components describing the various distributions were determined. Resampling was used for some of the simulated data to determine the significance of any observed differences between the conditional and unconditional distributions.

Analysis of the simulated data with limited time resolution indicated that the time

constants of the exponential components describing the various distributions were, within the limits of stochastic variation, independent of previous channel activity, whereas the areas of the components usually changed significantly; the observed (but insignificant) changes in time constants were typically <1–20%, whereas the changes in areas could be as large as 50–600%. For example, in a simulation of a model with three open and five shut states, none of the time constants of the exponential components in the four examined conditional distributions changed significantly from those in the unconditional distribution ($P > 0.22$–0.67), whereas the areas of 75% of the components in the four conditional distributions did change significantly ($P < 10^{-22}$ to 0.005).

Missed events in the simulated data did increase the time constants of some of the components as expected (Colquhoun and Sigworth, 1983; Blatz and Magleby, 1986a), but the increases were found to be the same for the conditional and unconditional distributions. Furthermore, provided that the fitting started at two times the dead time, as was done for the experimental data, in no case did missed events add erroneous components to either the unconditional or conditional distributions. However, tests of models in which several consecutive open and shut states were in series, such as —C—O—C—O—C—, indicated that in the absence of missed events some components in the unconditional distributions might not be detected in some of the conditional distributions, depending on the durations of the specified adjacent intervals. For these models, filtering and the resulting missed events often allowed the undetected components in some of the conditional distributions to be detected.

The above analysis of simulated data with limited time resolution suggests, then, that a finding for experimental data of exponential components with time constants independent of previous activity, but with dependent areas, would be consistent with Markov gating.

Conditional Distributions of Open Intervals

To test whether the activity of the BK channel is consistent with Markov gating, we examined the distributions of open intervals adjacent to specified shut intervals of different durations. Shut intervals from a record containing 62,580 intervals were sorted into classes based on their duration. Open intervals adjacent to the brief shut intervals (0–0.15 ms) are plotted in Fig. 3 A, and open intervals adjacent to the long shut intervals (>3 ms) are plotted in Fig. 3 B, as open circles in each case. Data from the open intervals before and after the specified shut intervals were combined, as they were not different when considered separately, as seen in Fig. 2.

The conditional open distributions shown in Fig. 3 were fit with the sums of exponentials (Eq. 3 in Methods). In each case the distributions were best described by four significant exponential components. The fit to the conditional open intervals adjacent to the brief shut intervals is plotted as a continuous line in Fig. 3, A and B, and the fit to the open intervals adjacent to the long shut intervals is plotted as a dashed line in Fig. 3, A and B. Fig. 3 shows that the form of the conditional distributions of open intervals depends on the duration of the adjacent shut intervals used to select the conditional distributions. The conditional distribution of open intervals adjacent to long shut intervals (*dashed lines*) contained a greater fraction of
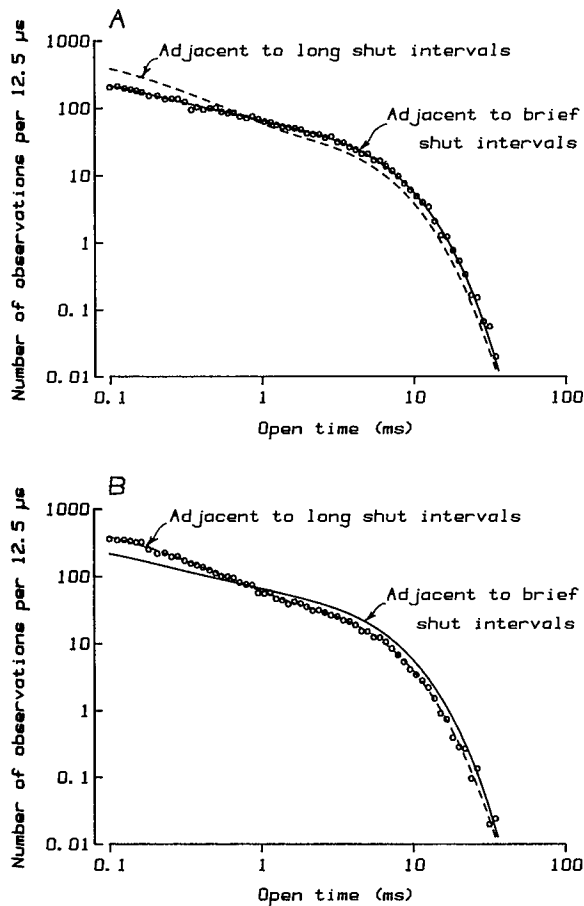
FIGURE 3. The conditional distributions of open times adjacent to brief (A) and long (B) shut intervals are plotted as open circles. For an experiment with 62,576 intervals, the open intervals adjacent to brief (0–0.15 ms) and long (>3 ms) shut intervals were binned into separate distributions and fitted with the sums of four exponential components. Each open interval can be binned twice, being adjacent to two different shut intervals. There were 25,196 open intervals fitted for A and 15,277 for B. The distribution in B was scaled to contain the same number of events (including those less than two times the dead time) as the distribution in A, to facilitate comparisons. In this and the following figures, the binned data are fitted and plotted starting at about two times the dead time to exclude the events whose durations are distorted by the limited time resolution. The continuous line in each plot is the most likely fit to the data in A, and the dashed line in each plot is the most likely fit to the data in B. The time constants (and areas) of the fitted distributions in A were: 0.098 ms (0.058), 0.42 ms (0.11), 3.3 ms (0.37), and 4.5 ms (0.46). The time constants (and areas) of the fitted distributions in B were: 0.097 ms (0.11), 0.36 ms (0.26), 3.5 ms (0.56), and 5.7 ms (0.07). There are relatively more brief open intervals adjacent to long shut intervals than adjacent to brief shut intervals. Effective low-pass filtering (24 dB/octave, −3 dB) of 4.0 kHz for a dead time of 45 μs. P_o = 0.18; membrane potential = 30 mV; Ca_i = 4.2 μM; pH 7.2.

brief openings and a smaller fraction of long openings than the conditional distribution of open intervals adjacent to brief shut intervals (*continuous line*).

The unconditional distribution of all open intervals from the same experiment was described by the sum of four exponentials, which was the same number as that used for the conditional distributions. The observation of four significant exponential components indicates that the BK channel enters a minimum of four open states in this experiment. Four open states is consistent with previous findings that the BK

channel typically enters three to four open states during normal activity (McManus and Magleby, 1988).

Time Constants of Conditional Open Distributions Are Independent of Shut Interval Durations

The time constants and areas of the exponential components describing the conditional distributions in Fig. 3, along with data obtained from a conditional distribu-
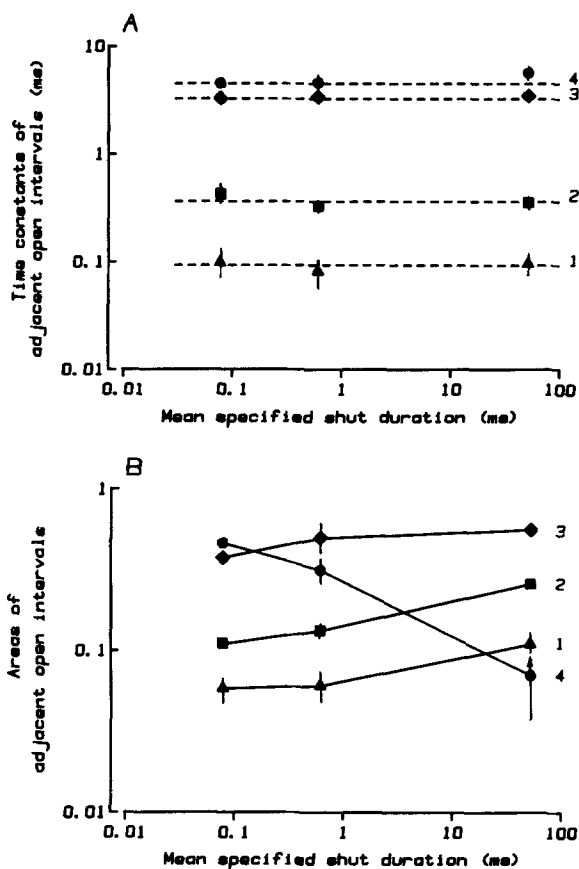

FIGURE 4. Time constants (*A*) and areas (*B*) of the exponential components describing conditional distributions of open intervals are plotted as a function of the mean durations of the adjacent shut intervals. Open intervals adjacent to brief (0–0.15 ms), intermediate (0.151–2.999 ms), and long (>3 ms) shut intervals were binned into separate distributions and fit with sums of four exponential components. The dashed lines in *A* plot the time constants of the four significant exponential components describing the (unconditional) distribution of all open times. The error bars are estimated standard deviations determined by resampling (see Methods). Error bars less than the symbol size are not plotted. The components are numbered based on their time constants, from briefest to longest. The time constants of the four components are independent of adjacent interval durations, and hence previous channel activity, whereas the areas of the components are dependent. Same experiment as Fig. 3.

tion of openings adjacent to shut intervals of intermediate duration (0.15–3.00 ms) are plotted against the mean duration of the specified adjacent shut intervals in Fig. 4 *A*. The exponential components are numbered sequentially, based on their time constants, from briefest to longest. The error bars in Fig. 4 plot estimated standard deviations for the time constants and areas describing the exponential components

in the three conditional distributions. These estimates, which were obtained by fitting artificial distributions resampled from the conditional distributions (see Methods), indicate the stochastic variability in the data and potential errors in the maximum likelihood fitting procedure. For some points the error bars are less than the symbol size and not visible.

T tests for the data in Fig. 4 A indicated that the time constants of the four exponential components in the three conditional open distributions were independent of the durations of the adjacent shut intervals, and were the same as the time constants (plotted as dashed lines) of the four components determined from the unconditional distribution of all open intervals.

In contrast to the time constants that remained unchanged, the areas of the exponential components describing the conditional open distributions were dependent on the adjacent shut interval durations. This is shown in Fig. 4 B, where the numbered components correspond to those in Fig. 4 A. The areas of components 1, 2, and 3 with the briefest time constants increased 90, 136, and 51%, respectively, and the area of component 4 with the longest time constant decreased 85%, as the mean adjacent shut interval increased from 0.078 to 52 ms.

A similar analysis was done for five additional data sets, each with 54,000–275,000 open and shut intervals during normal activity. The conditional open distributions from each data set were fit with the sum of four exponentials, the same number of significant components as in the distributions of all open intervals. The symbols in Fig. 5 plot the normalized medians of the time constants (A) and areas (B) of the exponential components from the conditional distributions for all six data sets (see Methods). The dashed lines in Fig. 5 A plot the mean time constants of the four components in the unconditional distributions of all open intervals for the six data sets. The error bars in Fig. 5 indicate the bidirectional dispersion of the median for the change of the time constants and areas of the exponential components describing the conditional distributions from those describing the unconditional distributions of all intervals for the six experiments (see Methods). Similar results were obtained when the normalized means of all six data sets were plotted.

Results from the six experiments analyzed for Fig. 5 indicate that the time constants of the conditional distributions are independent of the durations of the adjacent shut intervals. For example, a paired t test used to compare the time constants in the conditional distribution adjacent to the brief specified shut intervals with those adjacent to the long specified shut intervals, indicated no significant differences in time constants between the two distributions, independent of whether the t test was applied to normalized ($P > 0.1$–0.6) or unnormalized ($P > 0.2$–0.9) data (see Methods). The time constants of the conditional distributions were also not significantly different from the mean time constants of the unconditional distributions of all open intervals.

In contrast to the invariant time constants, the areas of components 1, 2, and 3 with the briefest time constants increased 96, 131, and 74%, respectively, and the area of component 4 with the longest time constant decreased 46%, as the mean adjacent shut interval increased from 0.043 to 41 ms in Fig. 4 B. A paired t test applied to the data before normalization indicated that the changes for components 1, 2, and 4 were significant ($P < 0.05$) with a P value for the observed change in

component 3 of 0.055. Paired t tests applied to the normalized data (to reduce the effect of variability among experiments) indicated that the areas of all four components changed significantly (see Methods).

Since the area of an exponential component reflects the number of intervals that make up that component, Figs. 4 and 5 show that the inverse relationship between adjacent open and shut intervals, as presented in Fig. 2 *A*, arises from a change in
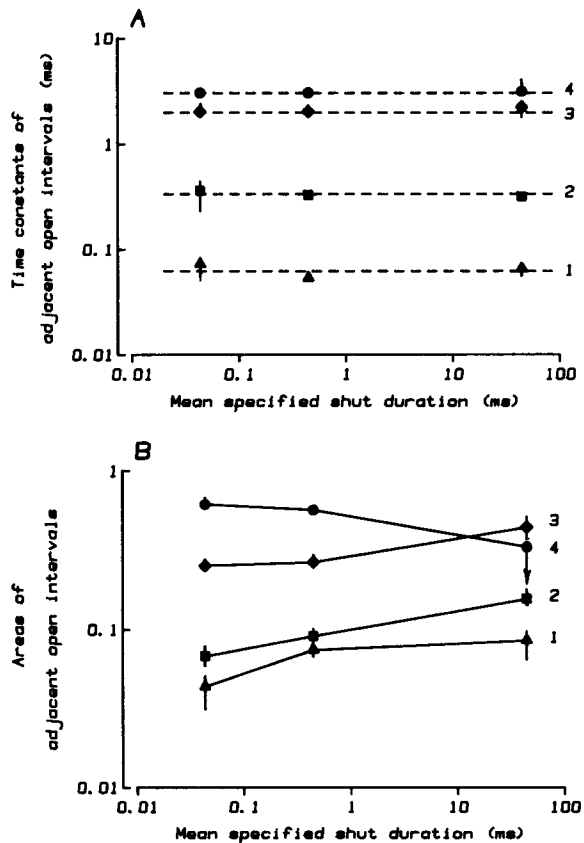

FIGURE 5. Median time constants (*A*) and areas (*B*) of the exponential components describing conditional open time distributions for six experiments. Open intervals adjacent to brief, intermediate, and long shut intervals were binned into separate distributions for each of the six data sets and fitted with sums of four exponentials. The time constants and areas were normalized (see Methods) to show the effect of adjacent interval durations on the changes in the areas and time constants rather than the absolute differences in values among the six different data sets. The error bars give a measure of the dispersion of the median, calculated separately in each direction, and include the middle two quarters of the observations (see Methods). In some cases the error bars are less than the symbol size and not visible. The lower and upper values of the error bar for the right most point of the area of component 4 are 0.17 and 0.79, respectively. Plots of the means and standard errors of the means gave similar results. The dashed lines in *A* plot the mean of the time constants determined from the unconditional distributions of all open times for the six data sets. Components are numbered from briefest to longest time constants. A total of 940,600 open and shut intervals were analyzed for the six data sets, which had P_o values of: 0.064, 0.18, 0.21, 0.47, 0.58, 0.70.

the relative numbers of intervals arising from the different exponential components, and not from a change in time constants.

The Conditional Open Distributions Are Consistent with Markov Gating

The combined results in Fig. 5 from six experiments are similar to the results of the single experiment shown in Fig. 4, and indicate that the time constants of the com-

ponents of the conditional open distributions are independent of the durations of the adjacent shut intervals. Since the observed time constants are determined by the underlying transition rates among the states, invariant time constants suggest rate constants independent of previous channel activity (Fredkin et al., 1985). Thus, analysis of the conditional open distributions suggests that gating of the BK channel is consistent with a Markov process.

Conditional Distributions of Shut Interval Durations

As a further test to determine whether Markov or non-Markov gating mechanisms are consistent with the kinetics of the BK channel, we examined the distributions of shut intervals conditional on the durations of adjacent specified open intervals. Results are shown in Fig. 6 where the conditional distributions of shut intervals adjacent to brief open intervals (0–0.8 ms, Fig. 6 A) and long open intervals (0.8–100 ms, Fig. 6 B) were fit with sums of eight exponential components, the number of significant exponential components in the unconditional distribution of all shut intervals.

The continuous lines in Fig. 6, A and B are the best fit sum of eight exponentials to the conditional distribution of shut intervals adjacent to the brief open intervals. The dashed lines are the best fit sum of eight exponentials to the conditional distribution of shut intervals adjacent to the long open intervals. On average, briefer shut intervals were more likely to occur adjacent to long open intervals than to brief open intervals.

The time constants and areas of the exponential components describing the conditional shut distributions in Fig. 6 are plotted against the mean durations of the specified adjacent open intervals in Fig. 7. The error bars indicate estimates of standard deviations, obtained with resampling (see Methods). The time constants of seven of the eight components of the two conditional shut distributions were independent of the durations of the adjacent open intervals, and were the same as the time constants of the components fit to the unconditional distribution of all the shut intervals (dashed lines). Only the longest time constant changed appreciably, but the change was not significant. The insignificant but apparent change in the longest time constant is consistent with the small numbers of events defining this component (only about 50 and 10 events for the conditional shut distributions adjacent to brief and long open intervals, respectively).

Whereas the time constants of the components in the conditional shut distributions were independent of the durations of the adjacent open intervals, the areas of some of the components were dependent (Fig. 7 B). The area of component 1 with the briefest time constant increased 25%, components 2 and 3 remained unchanged, and the areas of components 4–8 with the longer time constants decreased 21, 50, 35, 35, and 90%, respectively, as the durations of the mean adjacent open intervals increased from 0.29 to 2.8 ms.

Fig. 8 shows that similar results were obtained when data were analyzed from four experiments that had eight significant exponential components. These four experiments, plus two others that were not included because they contained fewer than eight significant shut components, are the same experiments analyzed for the analysis of the conditional open distributions in Fig. 5. Fig. 8 A shows that the time con-
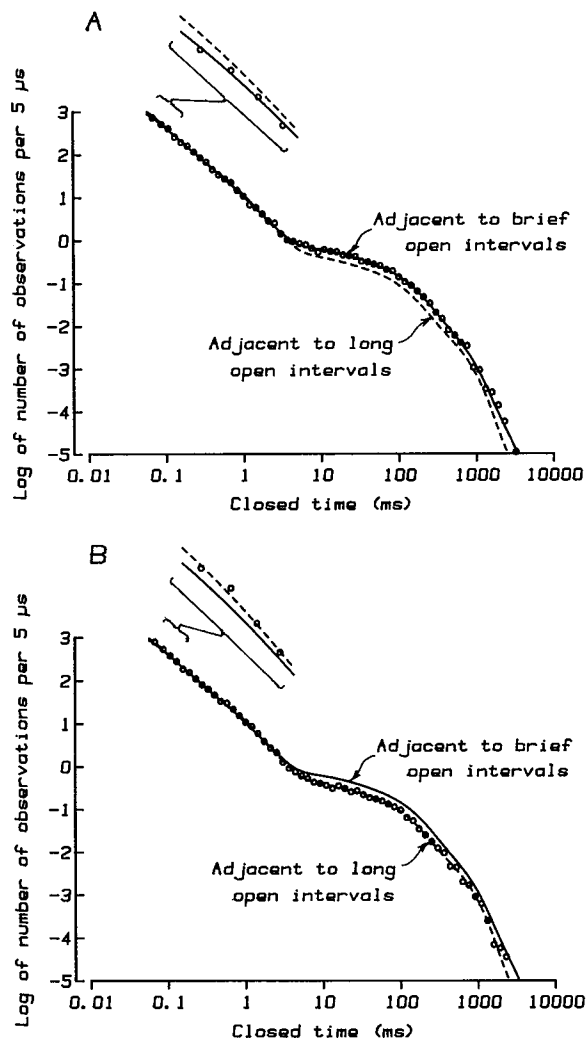
FIGURE 6. The conditional distributions of shut times adjacent to brief (*A*) and long (*B*) open intervals are plotted as open circles. For an experiment with 54,686 open and shut intervals, the shut intervals adjacent to brief (0–0.7 ms) and long (>0.7 ms) open intervals were binned into separate distributions and fitted with the sums of eight exponential components. Each shut interval was binned twice, as it was adjacent to two open intervals. There were 16,530 shut intervals fitted for the distribution in *A* and 23,518 fitted in *B*. The distribution in *A* was scaled to contain the same number of observed events (including events less than two times the dead time) as the distribution in *B*, to facilitate comparisons. The continuous line in each plot is the most likely fit to the data in *A*, and the dashed line in each plot is the most likely fit to the data in *B*. The inserts present enlarged plots of the indicated sections of the data, such that the entire ordinate and abscissa each now represent one log unit. The fitted time constants (and areas) for the distribution in *A* are: 0.033 ms (0.36), 0.089 ms (0.22), 0.29 ms (0.14), 0.96 ms (0.074), 13.8 ms (0.022), 76 ms (0.14), 274 ms (0.043), and 826 ms (0.0021). The fitted time constants (and areas) for the distribution in *B* are: 0.034 ms (0.45), 0.094 ms (0.22), 0.37 ms (0.14), 1.1 ms (0.058), 10.5 ms (0.011), 68 ms (0.091), 284 ms (0.028), and 2,050 ms (0.00021). There are relatively more long shut intervals adjacent to brief open intervals than adjacent to long open intervals. Effective low-pass filtering to give a dead time of 30 μs. P_o = 0.064; membrane potential = 30 mV; Ca_i = 5.9 μM; pH = 7.0.

stants of the eight components of the conditional shut distributions for the averaged data were independent of the durations of the adjacent open intervals, and were the same as the time constants of the eight components in the distributions of all shut intervals (*dashed lines*). Insignificant changes in the time constants were found by paired *t* tests, independent of whether the tests were applied to normalized ($P > 0.1$–0.7) or unnormalized ($P > 0.3$–0.9) data (see Methods).

In contrast to the unchanging time constants, the areas of the components describing the conditional shut distributions for the combined data often changed as a function of the duration of the adjacent open intervals, as shown in Fig. 8 B. The area of component 1 with the briefest time constant increased 20%, the areas of components 2–4 showed little change (4, 2, and −3%, respectively), and the areas of components 5–8 decreased appreciably (39, 43, 48, and 77%, respectively), as the duration of the adjacent open intervals increased from 0.33 to 3.8 ms. Paired t tests applied to the data before normalization indicated that the changes in the areas of
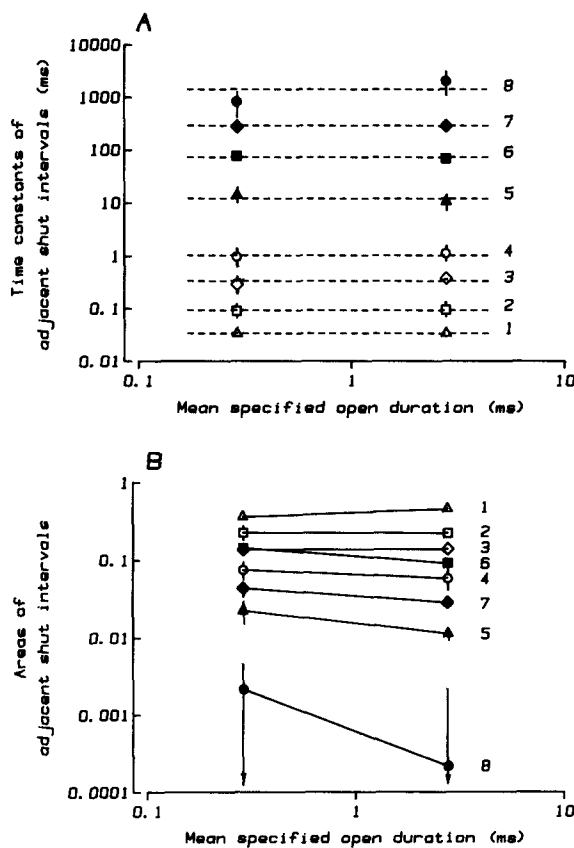

FIGURE 7. Time constants (A) and areas (B) of the exponential components describing the conditional shut time distributions in Fig. 6 are plotted as a function of the adjacent open time. The dashed lines in A plot the time constants of the eight significant exponential components describing the unconditional distribution of all shut times. The components are numbered based on their time constants, from briefest to longest. The error bars plot estimated standard deviations determined by resampling (see Methods). Standard deviations less than the symbol size are not plotted. The time constants of the first seven components are independent of adjacent interval durations, and hence previous channel activity, whereas the areas of some of the components are dependent. So few events are in the eighth component that the apparent change in the time constant is not significant. Same experiment as Fig. 6.

components 1 and 5 were significant. Paired t tests applied to the normalized data, to test for changes rather than absolute differences, indicated that the areas of components 1 and 5–8 changed significantly (see Methods).

The Conditional Shut Distributions Are Consistent with Markov Gating

The observations in Figs. 6–8 of conditional shut distributions with time constants independent of adjacent interval durations are consistent with rate constants inde-

pendent of previous channel activity and a Markov gating process (Fredkin et al., 1985).

Detection of Non-Markov Gating for a Specific Model

The previous sections indicated that the time constants for the components describing the conditional distributions were independent of the durations of adja-
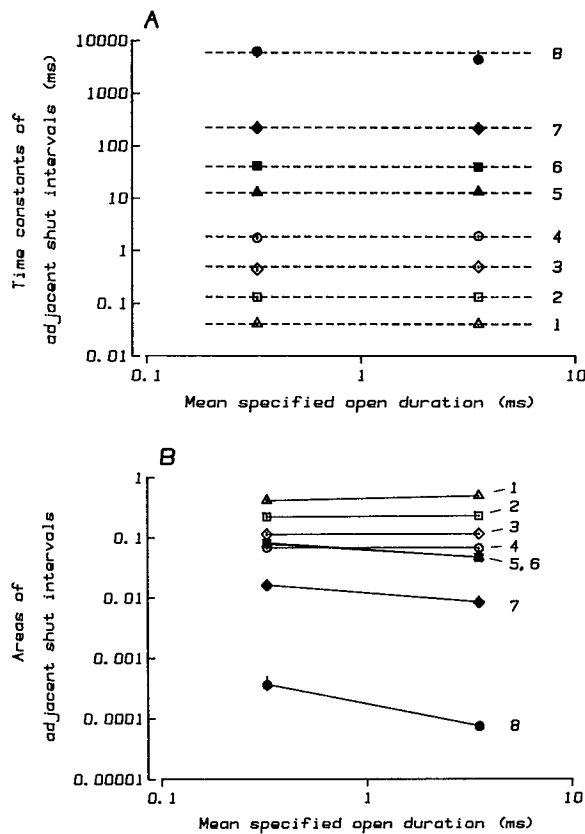
FIGURE 8. Median time constants (A) and areas (B) of the exponential components describing conditional shut time distributions from four experiments. Two conditional shut time distributions were obtained from each of the four experiments by binning shut intervals adjacent to brief and long open intervals separately. The conditional distributions were then fitted with the sums of eight exponential components. The time constants and areas were normalized (see Methods) to show the effect of adjacent interval durations on the changes in the areas and time constants rather than the absolute differences in values among the four different data sets. The error bars give a measure of the dispersion of the median, calculated separately in each direction, and include the middle two quarters of the observations (see Methods). For most of the plotted points, the error bars are less than the symbol size. Plots of the means and standard errors of the means gave similar results. The dashed lines in A plot the mean of the time constants determined from the unconditional distributions of all shut times for the four data sets. Components are numbered from briefest to longest time constant. A total of 625,500 open and shut intervals were analyzed for the four data sets, which had P_o values of 0.064, 0.18, 0.47, and 0.70.

cent intervals. The possibility arises, however, that our method of analysis might not detect changes in time constants that might arise from, for example, a non-Markov model such as scheme 2 with activity-dependent rate constants, even if such changes occurred.

To examine whether non-Markov gating could be detected for scheme 2 with

activity-dependent rates, intervals were simulated for a two state (C–O) non-Markov model in which the durations of the open and shut intervals were dependent on the duration of time spent in the previous state. The open and shut interval durations were calculated from the strictly empirical equations:

$$O = -\log{(Ran)}(1 \text{ ms}/S_p)^{0.5} \tag{10}$$

$$S = -\log{(Ran)}(1 \text{ ms}/O_p)^{0.5} \tag{11}$$

where O and S are the dwell times of the next sequential open and shut states, $-\log{(Ran)}$ (minus the natural log of a random number) gives exponentially distributed dwell times with a mean of 1.0 unit, O_p and S_p are the dwell times of the previous open and shut states, and the exponent in each equation determines the magnitude of the inverse relationship. An exponent of zero gives no dependence. Scheme
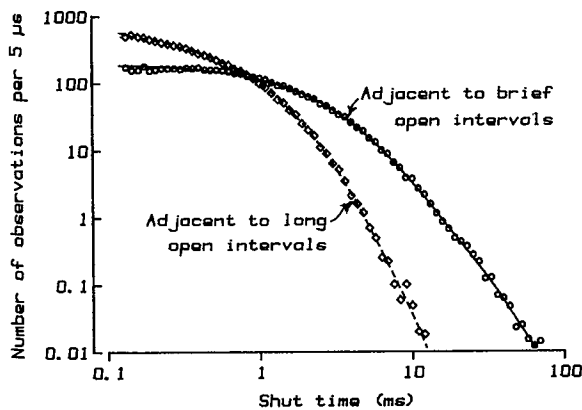

FIGURE 9. Conditional distributions of shut times for data simulated with the non-Markov model described by scheme 2 and Eqs. 10 and 11. For 200,000 simulated intervals, the shut intervals adjacent to brief (<0.54 ms) and long (>1.25 ms) open intervals were binned into separate distributions and fitted (for times >0.1 ms) with the sums of four (significant) exponential components. There were 76,036 fitted shut intervals adjacent to long open intervals and 58,311 adjacent to brief open intervals. The fitted time constants (and areas) for the distribution described by the continuous line through the circles are: 1.5 ms (0.55), 3.2 ms (0.38), 9.9 ms (0.067), and 55 ms (0.0032). The fitted time constants (and areas) for the dashed line through the diamonds are: 0.14 ms (0.10), 0.49 ms (0.55), 0.91 ms (0.33), and 2.2 ms (0.020). Similar values for the exponential components were found for 500,000 simulated intervals.

2 and Eqs. 10 and 11 were used to simulate 200,000 events, and a pronounced inverse relationship between the durations of open and shut intervals was observed (not shown). The simulated data were then analyzed to find the conditional distribution of shut intervals adjacent to open intervals <0.54 ms (Fig. 9, *open circles*) and the conditional distribution of shut intervals adjacent to open intervals >1.25 ms (Fig. 9, *diamonds*).

In contrast to the experimental data, in which the time constants were independent of previous channel activity, the time constants of the exponential components fitted to the conditional distributions for the non-Markov model described by scheme 2 and Eqs. 10 and 11 were highly dependent on previous channel activity. The time constants of the fitted exponentials describing the conditional distribution of shut intervals adjacent to open intervals <0.54 ms were: 1.5, 3.2, 9.9, and 55 ms, and the time constants of the fitted exponentials describing the shut intervals adja-

cent to open intervals >1.25 ms were: 0.14, 0.49, 0.91, and 2.2 ms. Such a difference in time constants between the two conditional distributions is consistent with the marked difference in slopes of the two conditional distributions in Fig. 9. Resampling indicated that the values of the fitted time constants were repeatable within ~±12% (SD), and similar fitted time constants were found for a separate analysis of 500,000 simulated events.

The non-Markov gating mechanism (scheme 2 and Eqs. 10 and 11) used to generate the data in Fig. 9 is simply an empirical model with no known physical basis. This analysis does show, however, that the methods used in this paper can detect data inconsistent with Markov gating for this specific non-Markov model. The key feature of this non-Markov model is that the rates for transitions between the open and shut states are dependent in a consistent manner on previous channel activity. Presumably, other non-Markov models with this same feature might also be detectable by the methods used in this paper. A strong dependence of the rates on previous activity was used for Fig. 9 (exponent of 0.5 in Eqs. 10 and 11). Further simulations showed that a much weaker dependence of the rates on previous activity for this specific non-Markov model could also be detected (exponent of 0.08 in Eqs. 10 and 11).

DISCUSSION

Markov Gating

Models for the gating of ion channels usually assume that the rate constants for transitions among a discrete number of kinetic states are independent of previous channel activity (Colquhoun and Hawkes, 1977, 1981; Neher and Stevens, 1977; Hille, 1984; Horn, 1984; Bezanilla, 1985). Although such discrete Markov models have been successful in describing channel gating, there has been little direct support for the basic assumption upon which these models are based. This paper examines the assumption of rate constants independent of previous activity by analyzing single-channel data from large conductance Ca-activated K channels (BK channels) in cultured rat muscle. The experimental approach is to examine the time constants of the exponential components describing conditional open and shut dwell-time distributions. If the observed time constants are independent of previous channel activity, then the underlying rate constants for the transitions among the kinetic states should also be independent of previous activity (Fredkin et al., 1985 and Eqs. 6–9). We find that the observed time constants of both open and shut conditional distributions are independent of previous channel activity, consistent with Markov gating.

Testing for Markov Gating

The test used for Markov gating is dependent on the ability to reliably estimate the time constants describing the exponential components in the dwell-time distributions. A first artifactual possibility for the observation of time constants independent of previous channel activity is that the analysis technique may not be capable of detecting changes in time constants, and hence changes in the underlying rate constants, even if they occur. This possibility can be rejected for several reasons: (a)

changes in Ca_i or membrane potential that change P_o (Barrett et al., 1982), and therefore must change some of the underlying rate constants, lead to detectable changes in some of the observed time constants (Magleby and Pallotta, 1983a, b). (b) Statistical analysis using resampling of the conditional distributions (see Methods) indicated that there was sufficient data to adequately define the time constants. Thus, any significant changes in time constants should have been detected (see limits of resolution below).

A second artifactual possibility for time constants independent of previous channel activity is that the previous channel activity may not have been different for the different conditional distributions. If the activity were not different, then the time constants would not be expected to change, even for activity-dependent rate constants. This possibility can be rejected since the areas of many of the various exponential components in the different conditional distributions did change significantly. A change in areas of the exponential components, but not time constants, indicates differences in previous channel activity for the different conditional distributions (Colquhoun and Hawkes, 1981; Fredkin et al., 1985; Eqs. 6–9).

The type of analysis presented in this paper requires tens to hundreds of thousands of intervals collected during stable activity. With fewer events than this there would not be sufficient data to adequately define all the exponential components (see McManus and Magleby, 1988). With unstable data, as might occur if some unknown factor were affecting the gating, such as possible interaction of the channel with the patch pipette or other proteins in the membrane, the time constants might be expected to change since the channel environment would be changing. Thus, an observation of changing time constants might be observed with Markov gating for non–steady-state conditions.

In any case, our observation of time constants independent of previous channel activity is not sufficient to globally reject non-Markov gating. Each non-Markov model of interest will have to be examined individually to determine whether it would generate data that could be distinguished from Markov gating. The techniques used in this paper were sufficient to detect non-Markov gating for at least the one examined non-Markov model (scheme 2 and Eqs. 10 and 11), and analysis of dwell-time distributions has been sufficient to show that the non-Markov (fractal) model considered by Liebovitch et al. (1987) is inconsistent with the experimental observations for four different ion channels (Korn and Horn, 1988, McManus and Magleby, 1988; McManus et al., 1988).

The tests used in this paper for Markov gating are restricted to channels in which there is a dependent relationship between adjacent open and shut dwell times. Without a dependent relationship, conditional distributions would not differ from the unconditional, and hence, the time constants describing the conditional and unconditional distributions could be the same, even for changing rate constants and non-Markov gating.

Other Evidence Consistent with Markov Gating

Support for Markov gating for the closing of acetylcholine receptor channels comes from the observation of Magleby and Stevens (1972) that end-plate currents decay with a time constant independent of the preceding holding potential, and hence the

previous channel activity. More direct support for Markov gating for the acetylcho-line receptor channel is the observation of Colquhoun and Sakmann (1985) of no asymmetry in bursts of openings. Closing of sodium channels in the absence of inactivation is also independent of previous channel activity. Hahin (1988) has found that, in the absence of inactivation, Na tail currents decay with two time constants independent of the preceding holding potential. The gating of fast Cl channels in muscle and gamma amino butyric acid–activated Cl channels in neurons also appears consistent with a Markov process (Blatz and Magleby, 1989; Weiss and Magleby, 1989).

Further support for Markov gating in BK channels comes from the observation of Pallotta (1985a) that the lifetime of a brief open state is independent of whether the channel inactivates, and hence previous channel activity. Additional, but more indirect evidence for discrete states and Markov gating in BK channels comes from observations of selective removal of single exponential components. Pallotta (1985b) found that N-bromacetamide selectively removes the exponential component with the longest time constant in the distributions of open intervals without affecting the time constants of the remaining open components. Such selective removal of one of the open components could easily be generated by discrete Markov models with multiple open states in which transitions to one or more of the open states become blocked by treatment with N-bromacetamide.

Restrictions on the Conclusions

Our findings that the time constants are independent of previous single-channel activity is restricted by the resolution and method of analysis. The plotted time constants do appear to be independent of previous activity in Figs. 4 A, 5 A, 7 A, and 8 A, but these are log plots which can obscure small changes. When data from a single data set (single channel, one experimental condition) were analyzed (Figs. 4 and 7), estimates of errors using resampling techniques indicated that the standard deviation of the percent error in estimates of the time constants averaged $\pm 13\%$ for the conditional open distributions and $\pm 20\%$ for the shut (excluding the shut component with the longest time constant, which was based on only a few events). Analysis of multiple data sets (Figs. 6 and 8) indicated that the normalized conditional time constants were typically within $< \pm 10\%$ of the unconditional time constants. The standard deviations of the time constants estimated from the decay of Na currents by Hahin (1988) were $\sim \pm 20\%$.

Thus, any changes in time constants as a function of previous channel activity are typically $< \pm 10$–20%. Since the time constants can depend in a complex manner on the transition rate constants (Colquhoun and Hawkes, 1977, 1981), possible changes in underlying rate constants could be more or less than this. Less than ± 10–20% of uncertainty is perhaps not too restrictive when compared with the two orders of magnitude separating the time constants of the fastest and slowest open exponential components and the five orders of magnitude separating the time constants of the fastest and slowest shut exponential components.

The conclusion of rate constants independent of previous channel activity for the BK channel applies only to activity in the normal mode, which includes 96% of the intervals (modes are characterized in McManus and Magleby, 1988). Tests for Mar-

kov gating during the other modes have not yet been made. Whether the transitions among the modes would best be represented by relatively simple models with time- or condition-dependent rate constants, or more complex models with time-invariant rate constants must await a more detailed understanding of the physical mechanisms underlying mode transitions.

Finally, the conclusion of rate constants independent of previous channel activity does not exclude the possibility that the rate constants may be continuously chang-ing at random. The analysis used in this paper would not detect such random changes, but only those correlated with the durations of the previous open and shut intervals. To investigate possible effects of random changes in rate constants, data were simulated for a two-state model in which the closing rate constant changed at random, with the range of change described by a normal distribution with a stan-dard deviation of ±10% of the mean rate. The open distribution of the simulated data was described by two significant exponential components with time constants 90 and 112% of the expected time constant for a fixed rate constant (45,000 open intervals). Increasing the standard deviation to 20% doubled the separation of the detected time constants. Methods will be needed to investigate whether rate con-stants change in a random manner, uncorrelated with previous channel activity, and if so, whether such changes give rise to some of the detected exponential compo-nents.

Discrete Markov Models and Protein Dynamics

Discrete Markov models derived from kinetic analysis of single-channel data are sta-tistical models. These models present the simplest kinetic schemes consistent with the experimental observations. The schemes consist of kinetic states and rate con-stants for the various transition pathways among the kinetic states. Discrete Markov models are useful to describe (and predict) the complex kinetics of channel gating, and also to calculate the contributions that different channels would make to cellu-lar function. Markov models also provide a means to explore the actions of various factors that modulate channel activity (e.g., Pallotta, 1985b) and provide a tool for exploring the relationship between observed kinetics and underlying structure (e.g., Sakmann et al., 1985).

Although there is an almost irresistable tendency to view the derived kinetic states as conformational states, such a one-for-one correlation does not have to be the case. For example, studies on protein dynamics suggest that proteins make rapid transition among a large number of conformational substates at physiological tem-peratures (reviewed by Frauenfelder et al., 1988). Conformational substates have slightly different molecular structures (local configurations) but similar energies, and have dwell times so brief that the full potential is explored in 10^{-8} s (Parak and Knapp, 1984). If channel proteins behave in a similar manner, then each kinetic state would reflect a large number of conformational substates. Furthermore, if the transition rates between conformational states are different, depending on the con-formational substates involved in the transition (see Frauenfelder et al., 1988), then the derived kinetic rate constants would represent average transition rates.

Although conformational substates of the type considered by Parak and Knapp (1984) appear far too brief to be directly detected by single-channel analysis, there

may well be additional conformational states with dwell times orders of magnitude longer than the conformational substates, but still too brief or with dwell times too similar for the conformational states to be detected as individual kinetic states by single-channel analysis. On this basis each of the detected kinetic states derived from single-channel analysis could represent one or more conformational states in rapid equilibrium, and each of these conformational states would represent a collection of conformational substates. (Theoretical studies can set restrictions on possible many-state Markov models [Läuger, 1988; Millhauser et al., 1988].)

At best, then, the discrete Markov models derived from single-channel analysis would only approximate the conformational states and protein motions associated with channel gating. Nevertheless, it appears that such discrete Markov models are sufficient to capture the physiologically relevant aspects of gating. For example, the numbers of major kinetic states determined from single-channel kinetic analysis for four different types of agonist or Ca-activated channels are consistent with the number of distinct states expected from Hill plots of the dose-response curves for the channels (Colquhoun and Sakmann, 1985; McManus and Magleby, 1988; Kerry et al., 1988; Weiss and Magleby, 1989).

Multiple Gateway States and Possible Transition Pathways

Once gating is shown to be consistent with a Markov process, then the observation of a dependent relationship between the mean durations of open and shut intervals (Fig. 2) or the observation of dependent conditional distributions (Figs. 3–8) requires that there be two or more independent transition pathways connecting open and shut states (Fredkin et al., 1985; McManus et al., 1985; Colquhoun and Sakmann, 1985). Scheme 1 (in Results) is an example of a model with four independent transition pathways. The observations in Figs. 2–8 that, on average, brief openings are more likely to occur adjacent to long shut intervals than brief shut intervals, would be consistent with models in which open states with briefer mean dwell times make direct connections with shut states with longer mean dwell times. If the dwell times of open states in scheme 1 increase from O_4 to O_1 and the dwell times of the shut states decrease from C_{10} to C_5, then scheme 1 could generate the necessary inverse relationship. However, the nonmonotonic decay often present in the relationship between adjacent open and shut intervals (Fig. 2 A), suggests that the relationship between the mean dwell times of the open and shut states is probably not so systematic.

The observation that all open components were observed adjacent to shut intervals of all (examined) durations would be consistent with transition pathways connecting the open states, as shown in scheme 1. However, the limited time resolution of the experiments could also generate apparent connections among open states if some of the transitions to intervening shut states were too brief to detect.

A further complicating issue in determining the transition pathways for the kinetic scheme for the BK channel is the observation of Pallotta (1985a) that at +50 mV BK channels inactivate almost exclusively from an open state with a brief lifetime. He found that 89% of the open intervals adjacent to the (inactive) shut intervals >200 ms had a mean dwell time of 0.15 ms. We have observed in some experiments on BK channels that there were mainly brief open intervals adjacent to longer

(inactive) shut intervals, as observed by Pallotta (1985a), and in other experiments that there are also intermediate and long open intervals adjacent to long shut intervals. For the membrane potential of +30 mV used for the majority of data collected for our paper, we have observed all open components adjacent to the longer shut intervals (Figs. 4 B and 5 B), and this was still the case for adjacent shut intervals longer than 200 ms. Thus, under some conditions, or for particular BK channels, there may be an additional inactive shut state entered almost exclusively from a brief open state. For other conditions or channels, such as presented in this paper, this pathway may be absent or much less prominent.

Apparent Thermodynamic Equilibrium

Stability plots (McManus and Magleby, 1988) indicated that the data analyzed for this paper were in a steady-state condition, but such plots do not exclude the possibility that channel gating may not be at thermodynamic equilibrium. The movement of ions through the channel could provide an energy source to drive the gating so that the kinetics would depend on the time direction of analysis (Colquhoun and Hawkes, 1983; Läuger, 1983, 1987; Colquhoun and Sakmann, 1985; Steinberg, 1987; Ball et al., 1988). We observed that the durations of those intervals immediately before and immediately after both open and shut intervals of a wide range of specified durations were not significantly different for the BK channel in 16 of 17 comparisons (Fig. 2). Since 1 out of 20 comparisons might be expected to be significantly different just due to chance alone, these observations are consistent with time reversal of gating kinetics for BK channels. Also consistent with time reversal is the observation of Pallotta (1985a) that the brief open intervals immediately before and after an inactive shut state had similar distributions.

A demonstration of a lack of time reversal in the single-channel current record would indicate a deviation from thermodynamic equilibrium. The observation of time reversal is consistent with thermodynamic equilibrium, but is not necessarily sufficient to rule out that the channel may not be at thermodynamic equilibrium. Simulations (unpublished) have shown that the type of analysis in Fig. 2 can be relatively insensitive to detection of deviations from thermodynamic equilibrium, depending on the assumed model and degree of deviation. Thus, although the gating of the BK channel appears consistent with thermodynamic equilibrium, further experiments are needed with greater electrochemical driving forces on K ions before the possibility of nonequilibrium gating can be entirely ruled out.

Comparison with Previous Work and Other Channels

Our observations of rate constants independent of previous channel activity, multiple independent gateway states, and the implications from the analysis that the briefer open states are likely to make direct transitions to the longer shut states, provide more support for some of the assumptions that have been used to study gating of BK channels (Wong et al., 1982; Magleby and Pallotta, 1983a, b; Moczydlowski and Latorre, 1983; McManus et al., 1985; Golowasch et al., 1986; Singer and Walsh, 1987). Our general findings for gating of BK channels also appear consistent with models for the gating of glutamate channels (Kerry et al., 1988) and acetylcholine-activated channels (Dionne et al., 1978; Jackson et al., 1983; Colquhoun and

Sakmann, 1985; Larbarca et al., 1985). Finally, the observation that single-channel data for the BK channel are consistent with time reversal (Fig. 2) and, hence, do not show a deviation from thermodynamic equilibrium, is similar to the observation of time reversal in the data from glutamate-activated channels (Kerry et al., 1988) and from acetylcholine receptor channels (Colquhoun and Sakmann, 1985). Thus, different channels appear to have some common gating features.

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