

Testing for Microscopic Reversibility in the Gating of Maxi K⁺ Channels Using Two-Dimensional Dwell-Time Distributions

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ABSTRACT An assumption usually made when developing kinetic models for the gating of ion channels is that the transitions among the various states involved in the gating obey microscopic reversibility. If this assumption is incorrect, then the models and estimated rate constants made with the assumption would be in error. This paper examines whether the gating of a large conductance Ca-activated K⁺ channel in skeletal muscle is consistent with microscopic reversibility. If microscopic reversibility is obeyed, then the number of forward and backward transitions per unit time for each individual reaction step will, on average, be identical and, consequently, the gating must show time reversibility. To look for time reversibility, two-dimensional dwell-time distributions of the durations of open and closed intervals were obtained from single-channel current records analyzed in the forward and in the backward directions. Two-dimensional dwell-time distributions of pairs of open intervals and of pairs of closed intervals were also analyzed to extend the resolution of the method to special circumstances in which intervals from different closed (or open) states might have similar durations. No significant differences were observed between the forward and backward analysis of the two-dimensional dwell-time distributions, suggesting time reversibility. Thus, we find no evidence to indicate that the gating of the maxi K⁺ channel violates microscopic reversibility.

INTRODUCTION

The gating of the pores of ion channels is often described with kinetic schemes that assume reversible transitions among a number of open and closed states (Neher and Stevens, 1977; Colquhoun and Hawkes, 1977, 1982, 1983; Hille, 1992). If the gating is at thermodynamic equilibrium, then the numbers of forward and backward transitions for each individual reaction step will, on average, be identical, so that the gating obeys the principle of microscopic reversibility (Colquhoun and Hawkes, 1983; Lauger, 1983; Steinberg, 1987a, b). The recording of currents from ion channels requires nonequilibrium experimental conditions to produce a net flux of the permeating ions through the channel pores. If the flux of ions were coupled to the gating of the channel to provide a source of energy, then the gating could violate the principle of microscopic reversibility, even if the activity of the channel were in a steady state (Colquhoun and Hawkes, 1983; Lauger, 1983; Kirber et al., 1985; Kijima and Kijima, 1987; Steinberg, 1987a, b). Such nonequilibrium gating has been demonstrated for a Cl⁻ channel from *Torpedo* electroplax, where an observed asymmetry in transitions between inactivated and conducting states was coupled to the transmembrane electrochemical gradient (Richard and Miller, 1990).

The purpose of the present paper is to investigate whether the gating of the large conductance Ca-activated K⁺ channel (maxi K⁺ channel) displays nonequilibrium processes. If the gating is at thermodynamic equilibrium, then the kinetic properties of the single-channel current record must, on av-

erage, show time reversibility, being independent of whether the record is analyzed forward or backward in time (Colquhoun and Hawkes, 1983; Steinberg, 1987a, b). The method we have used to look for time reversibility is to compare two-dimensional dwell-time distributions of adjacent open and closed intervals obtained from forward and backward analysis of the current record (Fredkin et al. 1985; Steinberg, 1987a, b). A difference between the forward and backward distributions would indicate a violation of microscopic reversibility. We have also analyzed two-dimensional dwell-time distributions of pairs of open intervals and also of pairs of shut intervals to extend the resolution of the method to special circumstances in which intervals from different closed (or open) states might have similar lifetimes. The distributions are found to be independent of whether the data are analyzed forward or backward in time. Thus, we find no evidence to suggest that microscopic reversibility is violated.

MATERIALS AND METHODS

Preparation and recording

Currents flowing through large-conductance Ca²⁺-activated K⁺ channels (BK or maxi K⁺ channels; Marty, 1981; Pallotta et al., 1981; Latorre et al., 1989) in surface membranes of primary cultures of rat skeletal muscle were recorded with the patch clamp technique (Hamill et al., 1981), as described previously (Pallotta et al., 1981; McManus and Magleby, 1988). Recordings were made from excised "inside-out" patches of membrane containing a single maxi K⁺ channel, as indicated by opening steps to a single main conductance level during prolonged observations at high open probability. Single-channel data have been recorded under a variety of experimental conditions. In most experiments, EGTA (typically 1 mM) was used to buffer the free Ca²⁺ concentration (details in McManus and Magleby, 1988), but in some experiments, EGTA buffer was not used. Currents were usually collected with 140–150 mM (symmetrical) KCl on both sides of the membrane, but in some cases, large concentration gradients for K⁺ were obtained by replacing the KCl in the pipette (extracellular surface) with 150 mM

Received for publication 17 August 1993 and in final form 29 March 1994.

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0006-3495/94/07/91/14 \$2.00

N-methyl-D-glucamine (NMDG) while having either 150 or 500 mM KCl at the normal intracellular surface. TES buffer (*N*-tris(hydroxymethyl)-methyl-2-aminoethane sulfonic acid) 2–5 mM was used in all solutions to hold the pH at either 7.0 or 7.2. Experiments were performed at room temperature (21–24°C). The membrane potential of the excised patches was held at +30 mV unless otherwise stated.

Measuring interval durations

Currents were recorded with an FM tape recorder during the experiments with a frequency response of typically 0–20 kHz. The tape speed was then slowed, typically 8–64 times, and the current was filtered further before being sampled by computer. The effective high frequency cutoff when considering the filtering from all components was typically in the range of 4–10 KHz (–3 dB, four pole Bessel filtering). Open and shut interval durations were measured from the sampled single-channel current records with half-amplitude threshold analysis, using appropriate precautions to prevent possible measuring artifacts, as detailed in McManus and Magleby (1988, 1989). The effective sampling periods used for the analysis ranged from 5 to 12.5 μ s.

Two-dimensional dwell-time distributions

A consequence of microscopic reversibility of gating is that the kinetics of the single-channel current record must, on average, be the same whether the record is read forward or backward in time (Colquhoun and Hawkes, 1983; Steinberg, 1987a, b). Three different methods based on two-dimensional dwell-time distributions were used to check for time-reversibility of gating kinetics.

Open-shut distributions

The theoretical basis of the first method has been presented by Fredkin et al. (1985), Kijima and Kijima (1987), and Steinberg (1987a, b). Two-dimensional dwell-time distributions of the durations of adjacent open and shut intervals were obtained by analyzing current records in the forward and in the backward directions. A difference between the two distributions would suggest that the gating kinetics are not reversible.

To generate the two-dimensional distributions of adjacent open and shut intervals, the logs of the durations of each open interval and the following shut interval were used to locate a bin on the *x*-*y* plane, with the *z* axis indicating the square root of the number of interval pairs in each bin (Magleby and Weiss, 1990). Log binning at 10 bins per log unit (McManus et al., 1987) and the Sigworth and Sine (1987) transform were used for clarity in the presentation of data that span many orders of magnitude in interval duration and frequency of observation. The arrows in Fig. 1 connect the pairs of intervals that would be binned for the forward (A) and backward (B) two-dimensional dwell-time distributions of adjacent open and shut intervals. For both forward and backward analysis, the durations of the open intervals are indicated on the *x* axis of the two-dimensional distributions and the durations of the shut intervals are indicated on the *y* axis. Notice that the backward binning could also be accomplished by binning in the forward direction all pairs of intervals consisting of a shut interval followed by an open interval. The two-dimensional dwell-time distributions were then smoothed by using a two-dimensional moving bin average with three bins per side, with the number of events in each bin weighted as the inverse of the distance from the central bin. Thus, the numbers of events in the four corner bins in the 3×3 moving array were multiplied by 0.707 before being added to the events in the other five bins of the moving array. The total was then divided by 7.828 (4×0.707 to weight the corner bins + 5×1 to weight the center and noncorner bins) to obtain the weighted average for the bin in the position of the center bin in the smoothed distribution. The process was then repeated for all bins in the unsmoothed distribution to obtain the values for the smoothed distribution. Plots were generated from the smoothed distributions with the program SURFER (Golden Software, Golden, Colorado).

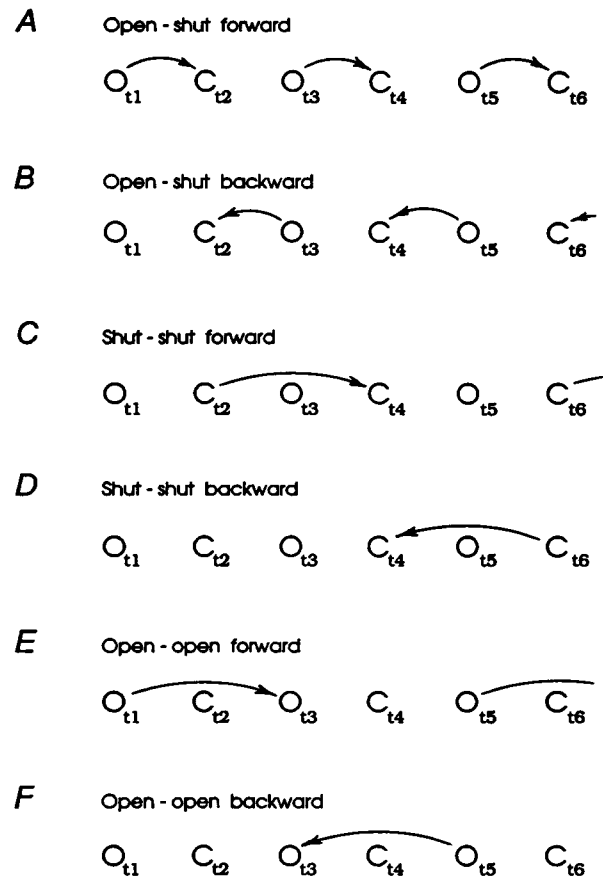


FIGURE 1 Selecting pairs of intervals for two-dimensional dwell-time distributions. The durations of consecutive open (O_n) and closed (C_n) intervals in the single-channel current record are indicated and numbered forward in time. The arrows indicate the pairs of intervals selected for the indicated two-dimensional dwell-time distributions (see Materials and Methods). The duration of the interval at the start of the arrow locates the event on the *x* axis, and the duration of the interval at the end of the arrow locates the interval on the *y* axis.

Shut-shut distributions

If the open intervals in a current record arise from several isolated open states with similar mean open times, then two-dimensional distributions of adjacent open and shut intervals, as described above, will not be useful for testing for microscopic reversibility because all of the open intervals will have the same mean durations, even if generated by different open states. However, if the closed states generate closed intervals of different mean closed times, then, as will be shown in Results, two-dimensional distributions of pairs of closed intervals can provide a means to investigate time reversibility. This is the second method that we have used to investigate microscopic reversibility. Different pairs of shut intervals are binned in both the forward and backward directions. A difference between the two distributions would suggest that the gating kinetics are not reversible. Fig. 1, C and D indicate the pairs of shut intervals that are binned in the forward and backward directions. The log of the duration of the first shut interval of each pair (e.g., C_{12} for forward binning and C_{16} for backward binning) is indicated on the *x* axis of the two-dimensional distribution, and the log of the duration of the second shut interval of each pair is indicated on the *y* axis. The *z* axis then indicates the square root of the number of shut interval pairs in each bin. Notice in Fig. 1, C and D that every other pair of shut intervals is used when binning in a given direction (i.e., C_{12} – C_{14} , C_{16} – C_{18} , ... for forward analysis of shut intervals and C_{10} – C_{12} , C_{14} – C_{16} , ... for backward analysis). Thus, similar to the binning of the open-closed intervals, all intervals are used in both forward and backward analysis, but the pairs are different for the different

directions. We also analyzed some of the data using every pair of intervals (i.e., C₁₂-C₁₄, C₁₄-C₁₆, . . . for forward analysis of shut intervals and C₁₆-C₁₄, C₁₄-C₁₂, . . . for the backward analysis), with similar findings to those obtained when every other pair was analyzed.

Open-open distributions

The third method used to investigate microscopic reversibility is the same as the second, except that two-dimensional distributions of pairs of open intervals rather than pairs of shut intervals are used. Fig. 1, E and F indicate the pairs of open intervals that are binned.

Simulated two-dimensional dwell-time distributions

Single-channel current records were simulated as detailed previously (Clay and DeFelice, 1983; Blatz and Magleby, 1986; Magleby and Weiss, 1990) and analyzed as described above to examine the ability of two-dimensional dwell-time distributions to detect deviations from microscopic reversibility in gating. When data were simulated for comparison with experimental results, the noise and filtering of the simulated current record were similar to that for the experimental current record.

Chi-squared test for significant differences between two distributions

χ² analysis was used to test for significant differences between the two-dimensional dwell-time distributions obtained by binning the current record in the forward and backward directions. Because it would be difficult to determine the appropriate degrees of freedom from smoothed distributions, the distributions used for the χ² calculations were not smoothed, but were binned at five bins per log unit to reduce variability. A χ² value was calculated from

$$\chi^2 = \sum_{i,j} \frac{[F(i,j) - E(i,j)]^2}{E(i,j)} + \frac{[B(i,j) - E(i,j)]^2}{E(i,j)}, \quad (1)$$

where F(i, j) and B(i, j) refer to the numbers of events in bin (i, j) for the forward and backward distributions, respectively, and E(i, j) is the average number of events in bin (i, j) for both the forward and backward distributions, given by

$$E(i, j) = [F(i, j) + B(i, j)]/2. \quad (2)$$

E(i, j) thus gives a measure of the expected number of events in bin (i, j) if the gating process were reversible. The summation in Eq. 1 is restricted to those bins with five or more events in both the forward and backward distributions, and the degrees of freedom are given by the number of pairs of bins that meet this criteria.

Because the degrees of freedom D are typically >100, it is necessary to use the approximate normal deviate Z to estimate significance, where

$$Z = \sqrt{2\chi^2} - \sqrt{(2D - 1)} \quad (3)$$

Observed differences are significant at the 5% level if Z > 1.96 (Snedecor and Cochran, 1991).

A series of comparisons of simulated two-dimensional distributions with numbers of events and degrees of freedom similar to those in this study indicated that the average differences between the numbers of observations in corresponding bins between two distributions had to be greater than about 5% before the χ² analysis indicated that the distributions were significantly different.

Calculating the fraction of transitions that are irreversible

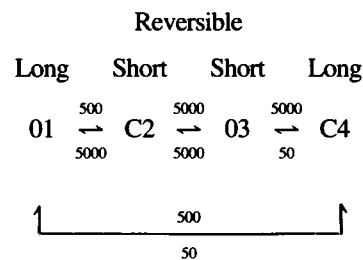
A difference in the product of the rate constants in the forward and backward directions around a loop in a kinetic model can indicate that the gating is

irreversible (Colquhoun and Hawkes, 1983), but the magnitude of the ratio of the product of the forward rate constants to that of the backward rate constants gives little information about the fraction of transitions in the gating that are irreversible. The reason for this is that rates approaching zero can have large effects on the ratio, but little effect on the actual numbers of transitions. The number of transitions per second from state A to state B, for example, in a kinetic scheme is given by the product of the equilibrium occupancy of state A times the rate constant for the transition from state A to state B. (Equilibrium occupancy is determined in the calculations described by Colquhoun and Hawkes, 1977, 1981.) Thus, if the equilibrium occupancy of state A is 0.1, (the channel spends 10% of its time in state A) and the rate constant from state A to state B is 1000/s, then the average number of transitions from state A to state B would be 100/s. Similarly, if the equilibrium occupancy of state B is 0.2 and the rate constant from state B to state A is 550/s, then the average number of transitions from state B to state A would be 110/s. Thus, of the 210 transitions/s between states A and B in this example, 10/s on average (4.8%) would be irreversible, reflecting the unequal transitions in each direction between states A and B. Similar calculations were carried out for all of the opening-closing transitions in each of various kinetic schemes to obtain the fraction of the total opening-closing transitions that were irreversible for each scheme. This fraction then served as a measure of the irreversibility of the gating.

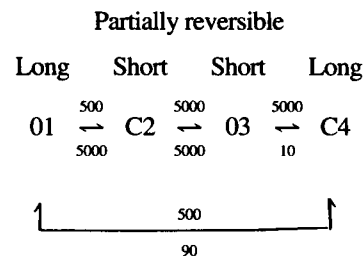
RESULTS

Detection of deviations from microscopic reversibility for simulated data

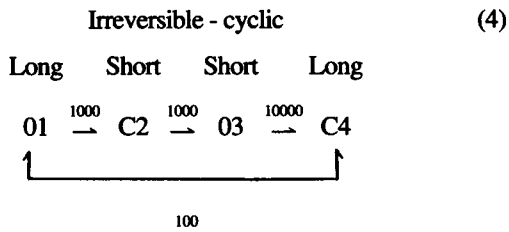
To investigate the ability of two-dimensional dwell-time distributions to detect deviations from microscopic reversibility in channel gating, we analyzed single-channel currents simulated for various gating mechanisms. Schemes 1-3 present three hypothetical models of channel gating, each with two open (O) and two closed (C) states connected in an alternating manner. The rate constants were selected for each scheme so that the mean lifetimes of the corresponding states are the same in the three different schemes. State O1 gives rise to a long open interval of 1 ms, O3 gives rise to a short open interval of 0.1 ms, C2 gives rise to a short closed interval of 0.1 ms, and C4 gives rise to a long closed interval of 10 ms.



Scheme 1



Scheme 2



Scheme 3

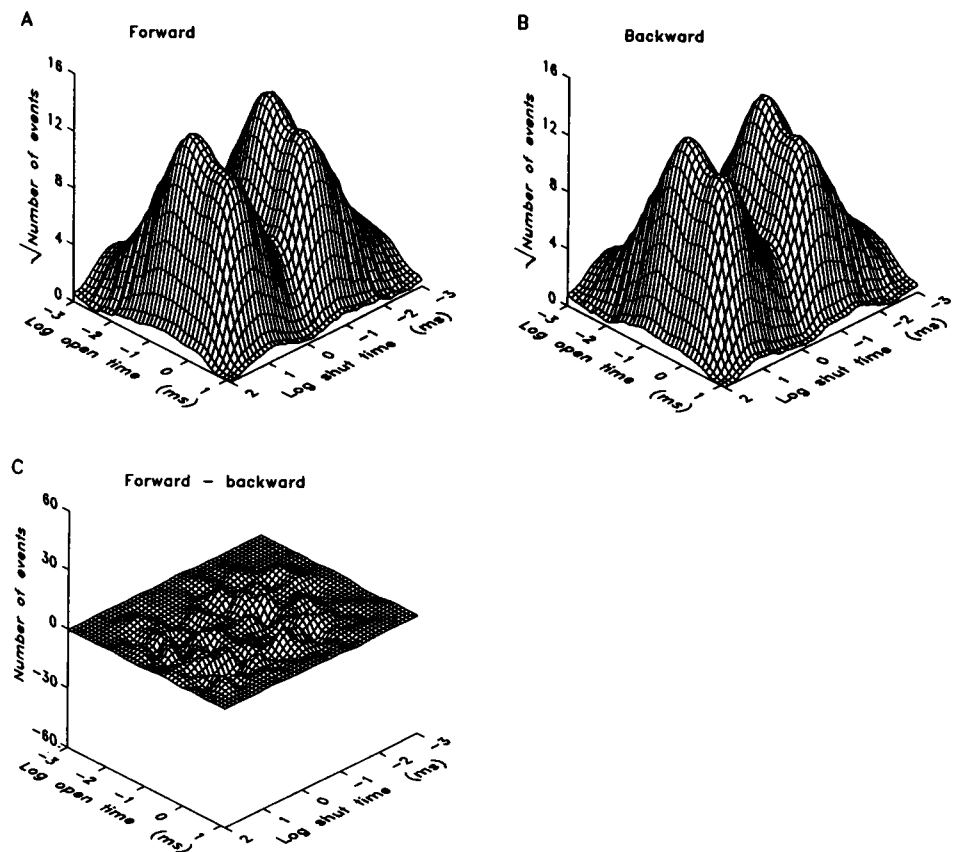
Scheme 1 is a completely reversible gating mechanism, with the product of the rate constants in one direction around the loop equal to the product in the opposite direction. Because the gating is reversible, there will be an equal number, on average, of transitions in both directions between each pair of connected states. For example, transitions between O1 and C2 will generate long open intervals followed by short closed intervals (O1-C2) and an equal number, on average, of short closed intervals followed by long open intervals (C2-O1). In a similar manner, transitions O1-C4 and C4-O1 will be equal in number, transitions O3-C2 and C2-O3 will be equal in number, and transitions O3-C4 and C4-O3 will be equal in number. Forward analysis of a single-channel current record for open intervals followed by closed intervals will detect all the open to closed transitions (O1-C2, O3-C2, O3-C4, and O1-C4), and backward analysis of the same record for open intervals that appear to be followed by closed intervals (because of the backward analysis) will detect all the closed to open transitions (C2-O1, C2-O3, C4-O3, and

C4-O1). Because the numbers of transitions in either direction between any two connected states are, on average, the same for reversible gating, then the two-dimensional distributions obtained from either forward or backward analysis of the current record must also be the same for reversible gating.

That this is the case for the reversible gating mechanism described by Scheme 1 is shown in Figs. 2, *A* and *B*, which present two-dimensional dwell-time distributions obtained by analyzing a simulated single-channel current in the forward and backward direction. Each distribution has four peaks that plot the numbers of transitions between the states: O1-C2, O1-C4, O3-C2, and O3-C4. The forward distribution plots the open-closed transitions between these pairs of states, and the backward distribution plots the closed-open transitions. The differences between the dwell-time distributions obtained from forward and backward analysis are shown in Fig. 2 *C*. The differences, which are small, reflect the expected stochastic variation in the simulated data. (Note that the differences are presented as absolute differences, whereas the two-dimensional distributions present the square root of the numbers of events.) Consistent with the lack of obvious visual differences, a χ^2 test indicated that the differences in Fig. 2 *C* were not statistically significant ($Z = -3.3$, see Materials and Methods).

Scheme 2 is a partially reversible gating mechanism, with the product of the rate constants in the clockwise direction

FIGURE 2 A reversible gating mechanism gives similar results for forward and backward analysis of the simulated single-channel current record generated with Scheme 1. (A) Two-dimensional dwell-time distribution for open-shut intervals obtained from the forward analysis of a simulated single-channel current record containing a total of 170,000 open and shut intervals. The *x* axis plots the log of the duration of the open interval in each open-shut interval pair, and the *y* axis plots the log of the duration of the shut interval in each open-shut interval pair selected as shown in Fig. 1 *A*. The *z* axis plots the square root of the number of interval pairs with the indicated durations. (B) Open-shut distribution obtained from backward analysis of the same current record analyzed for A. The open-shut interval pairs were selected as shown in Fig. 1 *B*. (C) Difference between the numbers of events in the open-shut distributions in A and B (A minus B). Note that the *z* axis is linear for the difference, rather than square root, as in A and B. There are no consistent differences in C, as expected for a model in which microscopic reversibility is not violated.



around the loop 9 times greater than the product of the rate constants in the counterclockwise direction. Fig. 3, *A* and *B* presents two-dimensional dwell-time distributions from forward and backward analysis of currents simulated with this partially reversible gating mechanism. Compared with the four peaks for Scheme 1 (Fig. 2, *A* and *B*), there are still four peaks for the forward analysis, but only three peaks for the backward analysis because the transition from C4 to O3 occurred so infrequently that it was not detected with the backward analysis. The pronounced differences between the forward and backward distributions (Fig. 3 *C*) were 10-fold greater than would be expected from stochastic variation alone (compare with Fig. 2 *C*) and were highly significant ($Z = 57$), clearly indicating that the gating violated microscopic reversibility.

To quantify the deviations from microscopic reversibility that are produced by the partially reversible gating mechanism described by Scheme 2, we calculated the rates (not rate constants) of each of the various transitions between the states in Scheme 2 (see Materials and Methods). Calculations of this type indicate that 30% of the total number of opening and closing transitions occur between O1 and C2, 20% occur between O2 and C3, 10% occur between O3 and C4, and 40% occur between O1 and C4. The fractions of each of these transitions that are irreversible is 16.7% between O1 and C2, 25% between O3 and C2, 10% between O3 and C4, and 12.5% between O1 and C4. Weighting the irreversible fractions for the numbers of opening and closing transitions be-

tween the various states indicates that, on average, 20% of the total opening-closings transitions are irreversible. As indicated above (Fig. 3 and the significant Z value), this level of irreversible gating is clearly detectable.

Fig. 4 presents results for the completely irreversible (cyclic) gating mechanism described by Scheme 3. Only two peaks are present in each distribution, and they are located in different positions for the forward and backward analysis because different transitions are detected for the different directions of analysis. As might be expected for a completely irreversible model, the differences between the forward and backward analysis are very large (Fig. 4 *C*, $Z = 338$), being 4–5 times greater than for the partially reversible model (compare with Fig. 3 *C*, noting the larger scale on the z axis in Fig. 4 *C*).

Figs. 2–4 show, then, that the observed differences between forward and backward analysis of the current record can detect violations of microscopic reversibility for the examined four state models.

Detecting deviations from microscopic reversibility for simulated data when the open states have similar lifetimes

If the open intervals arising from different open states have similar durations, on average, then two-dimensional analysis of open-shut intervals might not be expected to detect deviations from microscopic reversibility, because the open

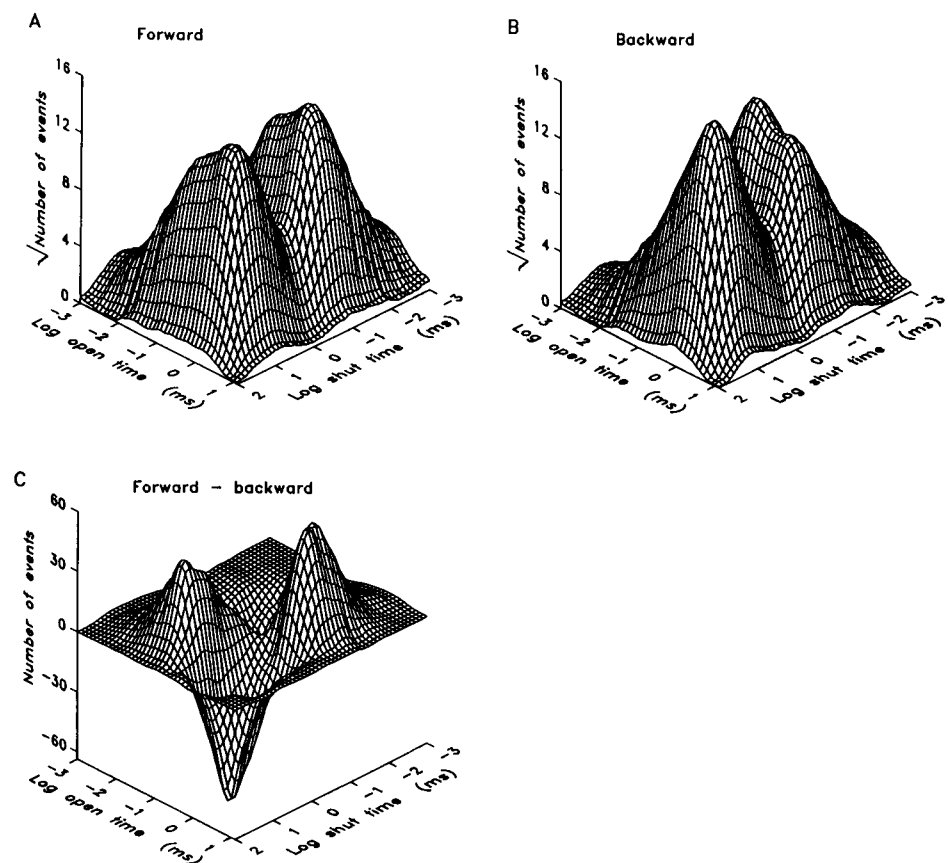


FIGURE 3 Detection of partially reversible gating for a single-channel current record simulated with Scheme 2. Except for the gating mechanism, the details are the same as for Fig. 2. The violation of microscopic reversibility is evident from the differences in *C*.

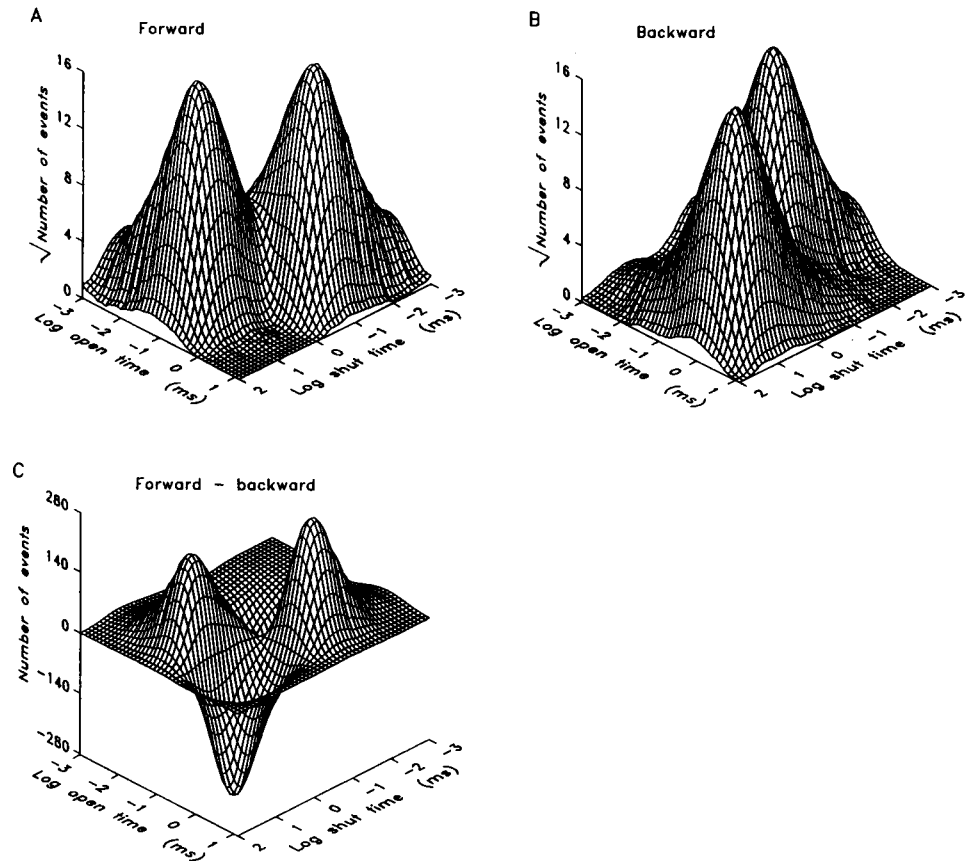
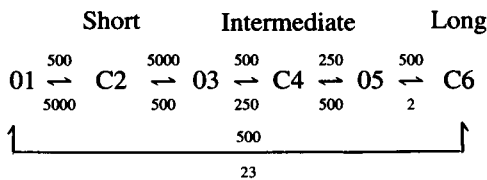


FIGURE 4 Detection of irreversible gating for a single-channel current record simulated with the cyclic gating mechanism described by Scheme 3. Except for the gating mechanism, the other details are the same as for Fig. 2. The violation of microscopic reversibility is evident from the marked differences in C.

intervals arising from different open states would be indistinguishable. This is shown in Fig. 5, where the data are generated with the six-state partially reversible gating mechanism described by Scheme 4, with the product of the rate constants in the clockwise direction being 11.5 times greater than the product in the counterclockwise direction. Fig. 5, A and B present the two-dimensional open-shut distributions for analysis of the current record in the forward and backward directions. Because the dwell times for the three open states are the same, only three peaks (instead of six) are present, reflecting the three different closed times of short, intermediate, and long lifetimes adjacent to the different open states with the same mean lifetimes. Even though Scheme 4 violates microscopic reversibility, the differences between the forward and backward analysis of the open-shut distributions are small, as shown in Fig. 5 C, and insignificant ($Z = -1.8$).



Scheme 4

Because the closed states for Scheme 4 have different mean lifetimes, the two-dimensional dwell-time distributions of pairs of closed intervals might reveal possible de-

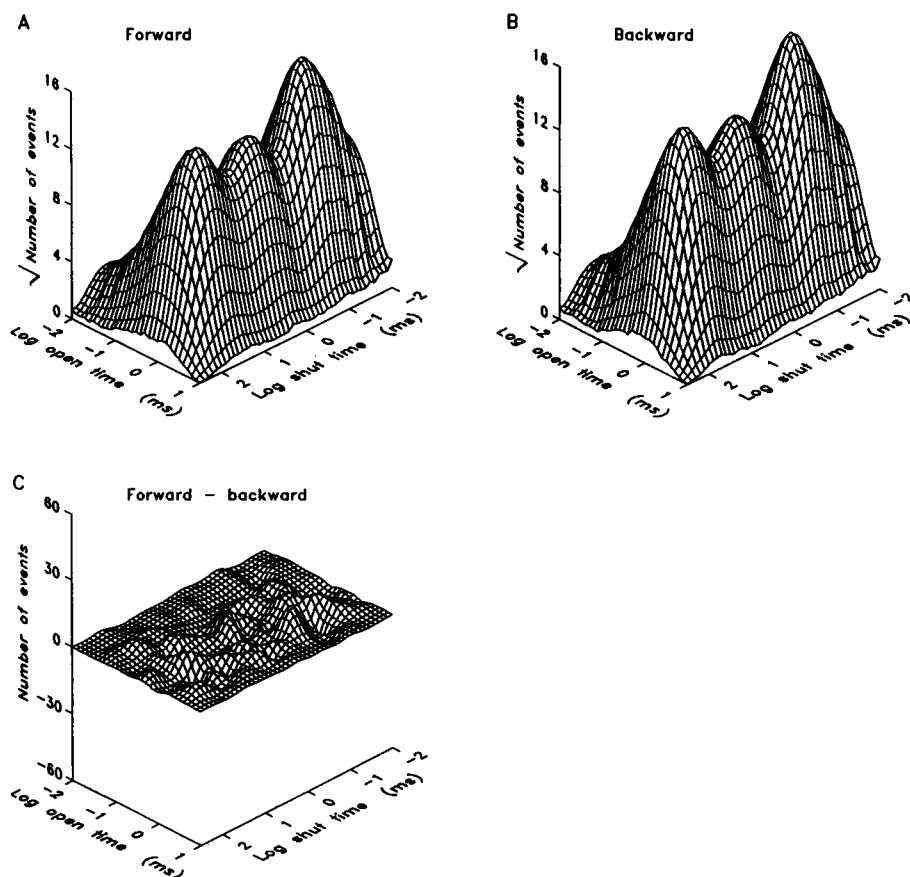
viations from microscopic reversibility. Fig. 6, A and B present the two-dimensional dwell-time distribution for pairs of shut intervals for Scheme 4, selected for forward and backward binning as shown in Fig. 1, C and D. In theory, nine peaks should be observable from the possible combinations of pairs of shut intervals: C2-C2, C4-C4, C6-C6, C2-C4, C4-C2, C4-C6, C6-C4, C6-C2, and C2-C6. Eight peaks (or partial peaks) are readily apparent, whereas the ninth peak (from C6-C4 in forward binning and C4-C6 in backward binning) is not visible because of its small magnitude.

If microscopic reversibility is obeyed, then the shut-shut distributions binned in the forward and backward directions in Fig. 6, A and B should be similar. The distributions are clearly different, and this significant difference ($Z = 32$), is emphasized in the difference plot in Fig. 6 C.

In contrast to the marked differences observed for the partially reversible six state model described by Scheme 4, there were only smaller insignificant ($Z = -2.3$) differences between forward and backward shut-shut distributions from a reversible model, as shown in Fig. 6 D-F. (The rate constants for the reversible model were the same as for Scheme 4, except that the rates for C6-O5 and C6-O1 were both 12.5/s.)

Thus, asymmetry in two-dimensional dwell-time distributions of shut-shut intervals can indicate violations of microscopic reversibility under certain conditions where open-shut distributions would not. Similarly, asymmetry in two-dimensional distributions of open-open intervals should also be a useful tool to test for violations of

FIGURE 5 Inability to detect partially reversible gating with open-shut distributions when the mean durations of the open states are identical for simulated single-channel currents generated with Scheme 4. (A) Two-dimensional dwell-time distribution for open-shut intervals obtained from the forward analysis of a simulated single-channel current record containing a total of 170,000 open and shut intervals. The open-shut interval pairs were selected as shown in Fig. 1 A. (B) Open-shut distribution obtained from backward analysis of the same current record analyzed for A. The open-shut interval pairs were selected as shown in Fig. 1 B. (C) Difference between the numbers of events in the open-shut distributions in A and B (A minus B). Even though microscopic reversibility was violated in the gating, there were no consistent differences for the forward and backward analysis.



microscopic reversibility under conditions where shut intervals arising from different shut states have similar lifetimes.

Lack of evidence for irreversible gating of the maxi K⁺ channel

Single-channel current records from excised patches, each containing a single maxi K⁺ channel, were analyzed to test for possible deviations from microscopic reversibility. Figs. 7 and 8 present results from the analysis of currents recorded with a large electrochemical gradient for K⁺, obtained with 500 mM K⁺ inside, zero K⁺ outside (details of solutions in Materials and Methods), and a membrane potential of +30 mV (inside positive). The open probability was 1.3%. The inset in Fig. 7 presents single-channel current records of two bursts of activity. The open-shut distribution from the forward analysis of over 54,000 open and closed intervals from this channel (Fig. 7 A) appears similar to the open-shut distribution from the analysis of the same data in the backward direction (B). Fig. 7 C shows that the differences between the forward and backward distributions are small, and the Z value of -3.58 indicates that the differences are not significant.

To examine whether stochastic variation would be sufficient to account for the differences in Fig. 7 C, we simulated a single-channel current record with noise and filtering similar to that of the experimental data (details in Blatz and

Magleby, 1986; Magleby and Weiss, 1990). The kinetic scheme used for the simulation had a reversible gating mechanism that could account for the major kinetic features of the channel (Scheme X in McManus and Magleby (1991), which is the same as Scheme 5 to be presented in the following section). The simulated current record was then analyzed in the same manner as the experimental record to obtain the differences plotted in Fig. 7 D. The differences for the data simulated with reversible gating were not significant ($Z = -2.23$) and were similar to the observed differences for the experimental data (compare Fig. 7, D and C), suggesting that stochastic variation in the gating is sufficient to explain the experimentally observed differences.

Fig. 8 presents the forward and backward analysis of the open-open (A and B) and shut-shut distributions (E and F) for the same experiment. The experimental differences between the forward and backward analysis were not significant for either the open-open (Fig. 8 C, $Z = -1.21$) or shut-shut distributions (Fig. 8 G, $Z = -2.96$), and were similar to those obtained from analysis of simulated data for the reversible model described above (Fig. 8, D and H).

Results of no evidence for deviations from microscopic reversibility in the gating were also found when a large electrochemical gradient was obtained with 150 mM K⁺ inside, zero K⁺ outside, and a membrane potential of +50 mV (176,000 open plus shut intervals analyzed).

Results similar to those in Figs. 7-8 were also obtained for seven additional maxi K⁺ channels, where the transmem-

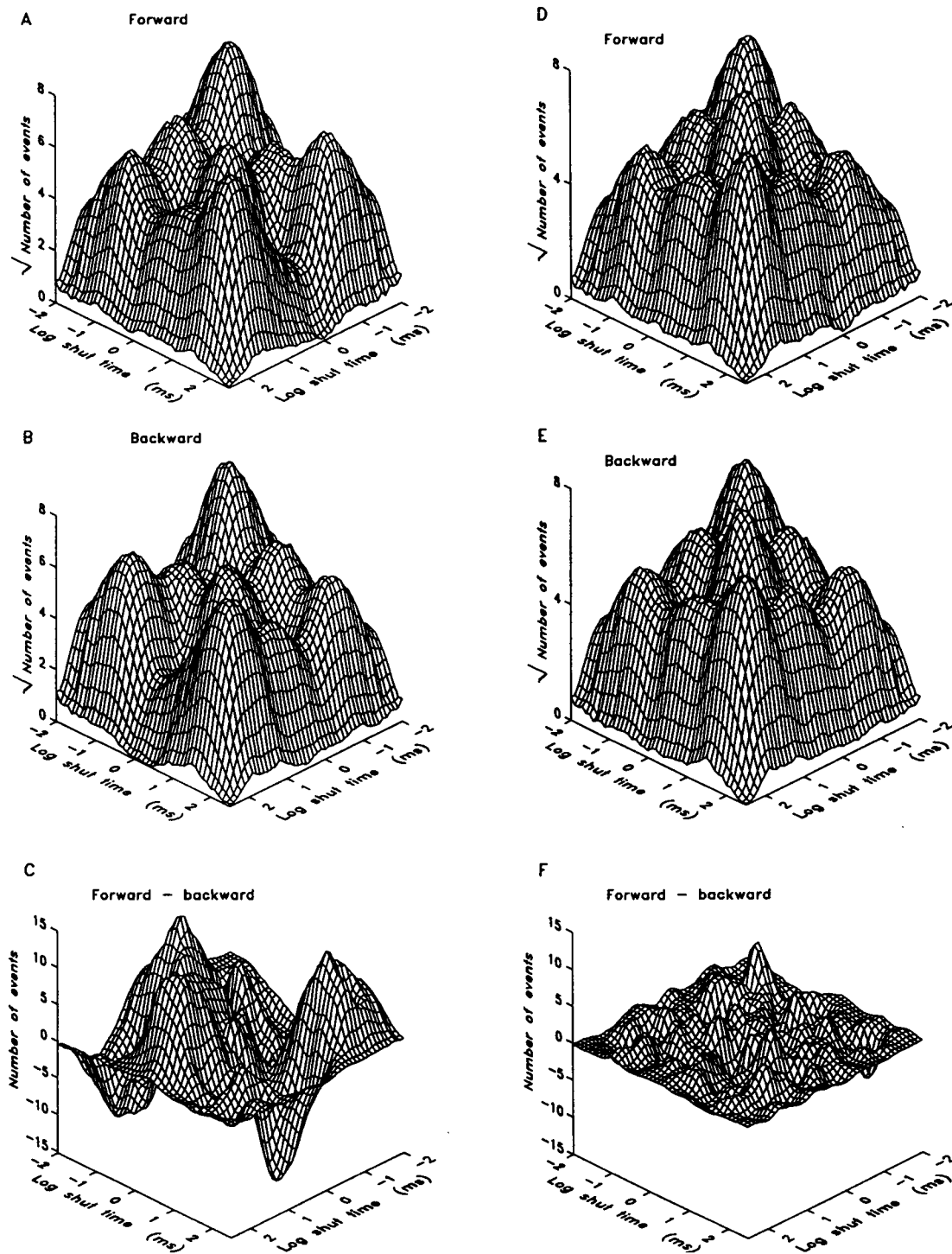


FIGURE 6 Detection of partially reversible gating with shut-shut distributions when the mean durations of the open states are identical for simulated single-channel currents generated with Scheme 4. (A and B) Two-dimensional dwell-time distribution for shut-shut intervals obtained from the forward (A) and backward (B) analysis of a simulated single-channel current record containing a total of 170,000 open and shut intervals. The shut-shut interval pairs were selected as shown in Fig. 1, C and D. (C) Difference between the distributions obtained by forward and backward analysis. The marked differences indicate irreversible gating. (D-F) Same as A-C, except that reversible gating was obtained by setting the rate constants for both transitions C6-O5 and C6-O1 to 12.5/s. No consistent differences were seen with reversible gating.

brane gradient of K^+ was symmetrical and a voltage gradient of +30 mV was applied to drive the K^+ through the channel. In these experiments, data were typically collected and analyzed for two different $[Ca^{2+}]_i$ for each channel. Open prob-

abilities ranged from 3 to 61%, and the numbers of analyzed intervals ranged from less than 10,000 to over 200,000. There was no evidence for deviations from microscopic reversibility in all cases, as indicated by insignificant Z values.

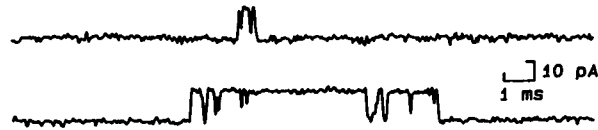
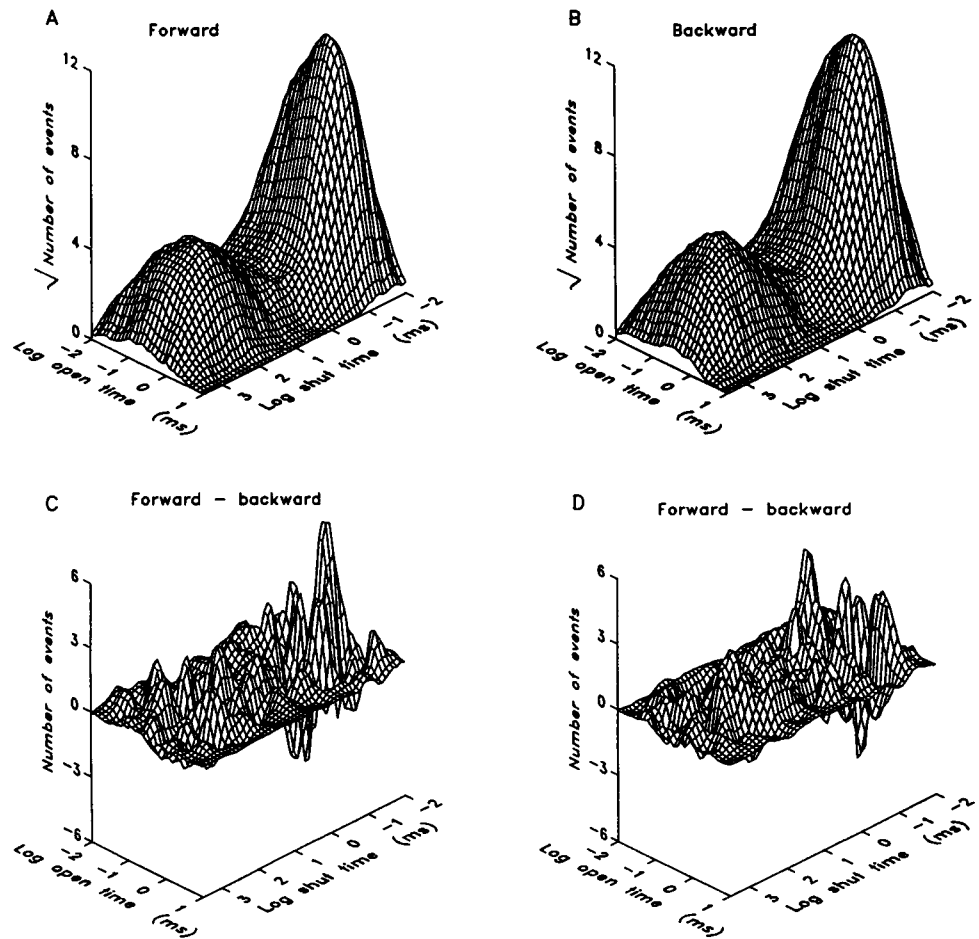


FIGURE 7 The gating of a maxi K⁺ channel, as revealed by open-shut distributions, is consistent with time reversibility. (*inset*) Selected bursts obtained from a maxi K⁺ channel in an excised patch of membrane with no added Ca²⁺ at the inner membrane surface. Opening is upwards. The open probability was 1.34%. A membrane potential of +30 mV (inside positive) with 500 mM K⁺ inside and zero K⁺ outside gave large outward currents (details of solutions in Methods). The effective filtering was 6.5 kHz (dead time of 0.028 ms). (*A* and *B*) Two-dimensional dwell-time distributions for open-shut intervals obtained from the forward (*A*) and backward (*B*) analysis of the single-channel current record; 54,254 open plus shut intervals were analyzed. The open-shut interval pairs were selected as shown in Fig. 1, *A* and *B*. (*C*) Difference between the numbers of events in the open-shut distributions in *A* and *B*. (*D*) Difference between the forward and backward analysis for 54,700 open plus shut intervals simulated with a reversible gating mechanism that could describe the data in Fig. 7*A*. The experimentally observed differences were similar to the simulated differences for the reversible model.



Some of the experimental data were also analyzed using dwell-time cross-correlation functions (Colquhoun and Hawkes, 1987; Ball et al., 1988). Such functions examine the correlation between open and shut intervals separated by an increasing number of intervening intervals. An example of forward (*solid line*) and backward (*dashed line*) cross-correlations for a maxi K⁺ channel with an open probability of 5.6% are shown in Fig. 9. These results are typical for what was observed in the cross-correlation analysis for six maxi K⁺ channels. There were no consistent differences between forward and backward analysis, and a negative correlation was present between the durations of open and shut intervals. The negative correlation was greatest for adjacent open and shut intervals and decreased as the number of intervening intervals increased. The negative correlation indicates an inverse relationship between the durations of open and shut intervals; long openings are often followed by short closings, and long closings are often followed by short openings, con-

sistent with previous observations (McManus et al., 1985; McManus and Magleby, 1988). The lack of differences in the cross-correlation functions obtained from forward and backward analysis of the data indicated no evidence for irreversible gating, consistent with the findings from the analysis of the two-dimensional dwell time distributions.

Placing limits on detectability of irreversible gating for the maxi K⁺ channel

The experiments in the previous sections found no evidence that microscopic reversibility was violated for the gating of the examined maxi K⁺ channels. The question arises, then, as to what extent microscopic reversibility would have to be violated before it would be detected by the methods used in this paper? This question can be approached, in part, by examining proposed gating mechanisms for maxi K⁺ channels. A minimal working hypothesis for the gating of the

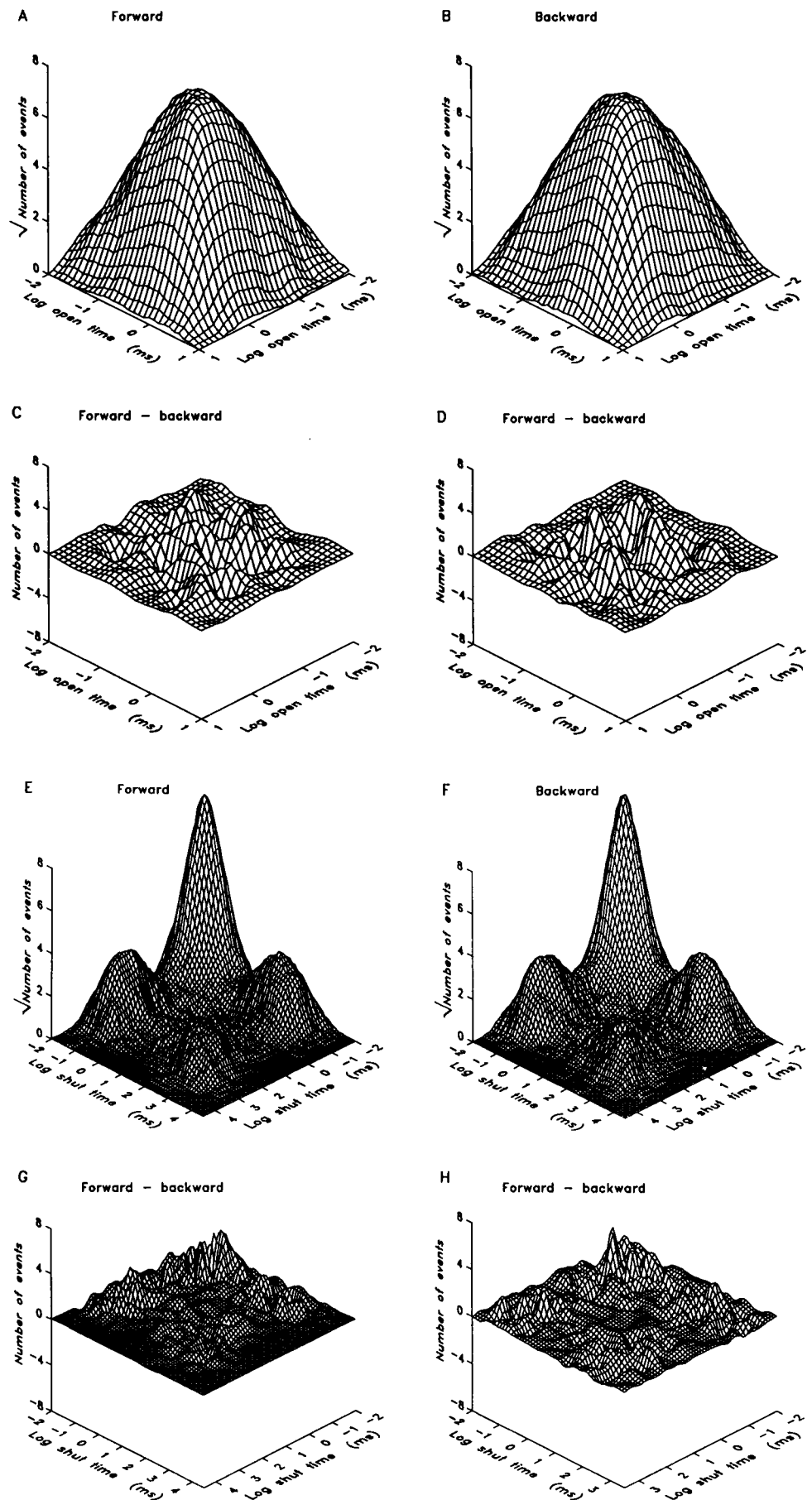


FIGURE 8 The gating of a maxi K^+ channel, as revealed by open-open and shut-shut distributions, is consistent with time reversibility. (*A* and *B*) Two-dimensional dwell-time distributions for open-open intervals obtained from the forward (*A*) and backward (*B*) analysis of the same single-channel current record analyzed for Fig. 7. The open-open interval pairs were selected as shown in Fig. 1, *E* and *F*. (*C*) Difference between the numbers of events in the open-open distributions in *A* and *B*. (*D*) Difference between the forward and backward analysis of open-open intervals simulated with a reversible gating mechanism that could describe the data in Fig. 7 *A*. The experimentally observed differences were similar to the differences for the simulated reversible model. (*E*–*H*) Same as *A*–*D*, except that the analysis is of pairs of shut intervals selected as shown in Fig. 1, *C* and *D*.

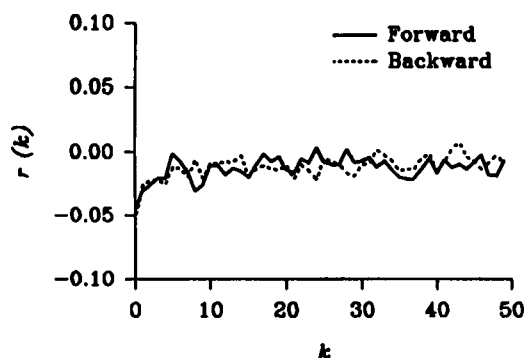
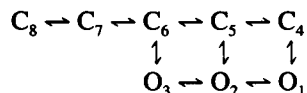


FIGURE 9 Dwell-time cross correlation functions, as defined in Ball et al. (1988), obtained from forward (—) and backward (- - -) analysis of a single channel current record obtained from a maxi K⁺ channel in an excised patch of membrane with 5.9 μM Ca²⁺ at the inner membrane surface and a membrane potential of 30 mV, inside positive. The value of the cross-correlation function, $r(k)$, is plotted against the lag, k , between open and closed intervals. When $k = 0$, the value of the cross-correlation function, $r(0)$, is for adjacent open and closed intervals. The effective filtering was 6 kHz (dead time of 0.030 ms), and 38,040 open plus shut intervals were analyzed. The data are consistent with time reversibility.

maxi K⁺ channel has been derived by the simultaneous fitting of multiple open and shut one-dimensional dwell-time distributions obtained at several different concentrations of Ca²⁺ (McManus and Magleby, 1991). The model, which has three open (O) and five closed (C) states, is shown without the Ca-binding steps in Scheme 5.



Scheme 5

Estimated rate constants for Scheme 5 are given in McManus and Magleby (1991) together with details of the fitting procedures. Scheme 5 has two loops: O1-O2-C5-C4-O1 and O2-O3-C6-C5-O2. Partially reversible gating could occur if the transitions around one or both of the loops were greater in one direction than in the other.

Because Scheme 5 differs in several aspects from the various gating mechanisms examined earlier, we first examined whether violations from microscopic reversibility for the gating for Scheme 5 could be detected by the methods used in this paper. Single-channel currents were simulated with Scheme 5 using rate constants selected to enhance the ability to detect partially reversible gating. Analysis of the simulated currents indicated that Scheme 5 with selected rate constants could produce detectable differences between the open-closed two-dimensional dwell-time distributions obtained from forward and backward analysis of the simulated current records. For example, a significant difference ($Z = 10.3$) was found between forward and backward analysis of pairs of open and shut intervals simulated with Scheme 5 using the following rate constants (s^{-1}): 1-2 = 90; 1-4 = 90; 2-1 = 5000; 2-3 = 5000; 2-5 = 5000; 3-2 = 500; 3-6 = 500; 4-1 = 500; 4-5 = 10; 5-2 = 5000; 5-4 =

5000; 5-6 = 5000; 6-3 = 90; 6-5 = 10; 6-7 = 10; 7-6 = 7; 7-8 = 8; 8-7 = 2.

To investigate the magnitude of the deviations from microscopic reversibility that were produced by the rate constants in this hypothetical example, we calculated the rate of each of the transitions between the open-closed pairs of states in the two loops in Scheme 5 (see Materials and Methods). Calculations of this type using the rate constants listed above indicated that 57% of the total number of opening and closing transitions occurred between O1 and C4, 13% occurred between O2 and C5, and 30% occurred between O3 and C6. The fraction of opening and closing transitions between O1 and C4 that was irreversible was 6%, the fraction that was irreversible between O2 and C5 was 46%, and the fraction that was irreversible between O3 and C6 was 8%. Weighting these fractions for the total number of opening and closing transitions indicated that, on average, 12% of the total opening-closing transitions were irreversible. Therefore, 12% irreversibility was sufficient to produce detectable deviations from microscopic reversibility for Scheme 5 for this specific example. In another example, 6% irreversibility was insufficient to produce detectable deviations from microscopic reversibility ($Z = -0.89$). Thus, to a first approximation the limit of detectability for violation of microscopic reversibility for Scheme 5 using forward and backward analysis of the single-channel data falls between 6–12% of the total opening and closing transitions being irreversible.

Having found that Scheme 5 with selected rate constants can produce detectable deviations from microscopic reversibility, we next explored whether Scheme 5 could give a better description of the experimental data if the rate constants were not constrained to produce reversible gating. Scheme 5 was simultaneously fitted to multiple one-dimensional dwell-time distributions, as described in McManus and Magleby (1991), except that the rate constants in the loops were no longer constrained to maintain microscopic reversibility. The most likely rate constants obtained when irreversible gating was allowed did not improve the fitting of the experimental data (based on maximum likelihood estimates) and were essentially the same as the rate constants obtained when the fitting was constrained to maintain microscopic reversibility.

The findings in the previous sections suggest, then, that if irreversible gating is present, it must be less than 6–12% of the total number of opening-closing transitions or it would have been detected, provided that the gating of the channel is similar to Scheme 5). The findings also indicate that reversible gating is sufficient to describe the gating kinetics, because allowing irreversible gating did not improve the fits. These observations are thus consistent with reversible gating, but they do not exclude the possibility that there might be some (less than 6–12%) irreversible gating.

To explore this possibility, we examined whether a set of rate constants could be found for Scheme 5 that would be consistent with the single-channel gating of the maxi K⁺ channel while giving some irreversible gating. Scheme 5 was simultaneously fitted to multiple one-dimensional

dwelling-time distributions as described in McManus and Magleby (1991), except that one or more of the rate constants in the loops in Scheme 5 were constrained to produce some irreversible gating. The likelihoods of the fits with various amounts of irreversible gating were then compared with the fits with reversible gating. Scheme 5 gave significantly worse descriptions of the experimental data (as determined by the likelihood ratio test; see McManus and Magleby, 1991) when the fraction of irreversible opening and closing transitions exceeded about 2–4% of the total number of opening and closing transitions. Thus, if Scheme 5 describes the gating of the maxi K⁺ channel, then the upper limit on the fraction of irreversible opening-closing transitions for the maxi K⁺ channel is less than about 2–4%.

DISCUSSION

No evidence for irreversible gating for the examined maxi K⁺ channels

If the gating of an ion channel is reversible so that each individual reaction step proceeds, on average, at the same rate in each direction, then the gating is at thermodynamic equilibrium and displays microscopic reversibility (detailed balance). A consequence of microscopic reversibility is that the gating must show time reversibility so that the kinetics of gating, on average, are independent of whether the single-channel current record is analyzed forward or backward in time (Colquhoun and Hawkes, 1983; Fredkin et al., 1985; Steinberg, 1987a, b). The results of our study indicate that the kinetics of the gating of maxi K⁺ channels, as revealed in two-dimensional dwelling-time distributions and in dwelling-time cross-correlation functions, are independent of whether the single-channel current record is read forward or backward in time. Thus, we find no evidence to suggest that the flux of K⁺ through maxi K⁺ channels drives the gating in an irreversible manner. These findings are consistent with previous observations obtained with less detailed analysis (Pallotta, 1985; McManus and Magleby, 1988).

The emphasis of our study was on the durations of open and closed intervals at the half-amplitude level to focus on the major opening and closing transitions for gating. Thus, if an irreversible passage through subconductance levels of the type observed infrequently for acetylcholine receptors (Hamill and Sakmann, 1981) were present, it would probably not be detected by our analysis. Maxi K⁺ channels do enter subconductance levels, however (Barrett et al., 1982; Rae et al., 1990; Tyerman et al., 1992; Ferguson et al., 1993), so that irreversible subconductance transitions might be possible. Nevertheless, the observation that averaged opening and closing transitions of current records for maxi K⁺ channels are typically mirror images of one another (Ferguson et al., 1993) would suggest that irreversible transitions through subconductance levels do not dominate the opening and closing kinetics.

Limits on the fraction of irreversible gating transitions

Whether irreversible gating would be detected from analysis of single-channel data is highly dependent on the specific gating mechanism and relative rate constants (Steinberg, 1987a, b; Ball et al., 1988). Because the actual gating mechanism of the maxi K⁺ channel is not known, the observation made in this study of no significant differences in kinetics for forward or backward analysis of the data does not necessarily rule out the possibility that some of the transitions in the gating of maxi K⁺ channels are fully or partially irreversible.

Although it is not possible to prove that a given model actually describes the underlying gating mechanism of a channel, Scheme 5 (see Results) is consistent with many of the observed kinetics of the maxi K⁺ channel. If Scheme 5 appropriately describes the underlying gating mechanism of the channel, then it is possible to place some upper limits on the fraction of irreversible gating. Analysis of simulated data generated using Scheme 5 with rate constants constrained to produce various deviations from microscopic reversibility indicated that 6–12%, on average, of the total number of opening and closing transitions would have to be irreversible for violations of microscopic reversibility to be detected by the methods used in this study. Because we did not detect any deviations from microscopic reversibility, then this observation places an upper limit of from 6–12% on the number of irreversible openings and closings. Further analysis then suggested that the fraction of irreversible transitions, if any, is considerably smaller than this. Fitting Scheme 5 to the multiple one-dimensional distributions of open and closed intervals while constraining the rate constants to produce various amounts of irreversible gating indicated that Scheme 5 gave significantly worse descriptions of the experimental data when the fraction of irreversible opening and closing transitions exceeded about 2–4% of the total number of opening and closing transitions. Thus, if Scheme 5 describes the underlying gating mechanism of the channel, then the fraction of irreversible opening and closing transitions for the maxi K⁺ channel must be less than about 2–4%.

In retrospect, it is perhaps not surprising that imposing significant amounts of irreversible gating on Scheme 5 produced worse fits to the experimental data. The usual pathway for gating of the maxi K⁺ channel with Scheme 5 and the most likely rate constants involves mainly transitions among the linearly connected states: C8-C7-C6-C5-O2-O1 (McManus and Magleby, 1991). Because significant transitions around loops are required for irreversible gating, and because previous studies suggested that the gating does not normally involve significant transitions around loops, it might be questioned why tests for irreversible gating were performed in the first place. The reason is that Scheme 5, like all kinetic models of complex phenomena, is probably not correct in specific detail, although it describes the general features of the gating. An experimental observation of irreversible gating kinetics would have indicated that Scheme 5

or its rate constants need modification to produce more pronounced loops in the gating process. The actual experimental observation of no evidence for irreversible gating lends further support, albeit rather indirect, for Scheme 5 and its derived rate constants.

It cannot be ruled out, however, that Scheme 5 is still too simple or that an entirely different scheme that produces significant, but undetectable, deviations from microscopic reversibility describes the actual gating of the maxi K⁺ channel.

Comparison with other channels

Richard and Miller (1990) found that the gating for a Cl⁻ channel from *Torpedo* electroplax was not at thermodynamic equilibrium even though channel activity was in a steady state. The number of transitions per unit time between conducting and inactivated states was unequal when the data were analyzed in forward and backward directions, and the asymmetry increased with the transmembrane electrochemical gradient for Cl⁻ ions. Thus, in contrast to findings for the maxi K⁺ channel, the gating for a Cl⁻ channel in electroplax appears to be coupled to ions flowing down their electrochemical gradient.

Colquhoun and Sakmann (1985) found for the acetylcholine receptor in skeletal muscle that the durations of successive open intervals during bursts displayed time reversibility. Kerry et al. (1988) found for glutamate receptors that the mean durations of adjacent intervals were independent of whether the intervals occurred immediately before or immediately after an interval of specified duration. Thus, similar to the gating for maxi K⁺ channels, the gating for both glutamate receptors and for bursts of acetylcholine receptors appears to be time-reversible.

Models for the gating of maxi K⁺ channels are usually based on kinetic schemes with transitions among a discrete number of states. The assumption of microscopic reversibility is usually made when estimating rate constants for the transitions, such that the number of transitions per unit time between directly connected states is, on average, the same in each direction. The results of this study are consistent with this assumption.

We thank Dr. Owen McManus for providing some of the data that were analyzed for this study, and Dr. W. Ferguson for carrying out some of the preliminary analysis.

This work was supported in part by grants to K. L. Magleby from the National Institutes of Health (AR-32805) and the Muscular Dystrophy Association. L. Song was supported in part from a Neuroscience training grant from the National Institutes of Health (NS07044).

REFERENCES

- Ball, F. G., C. J. Kerry, R. L. Ramsey, M. S. P. Sansom, and P. R. N. Usherwood. 1988. The use of dwell time cross-correlation functions to study single-ion channel gating kinetics. *Biophys. J.* 54: 309-320.
- Barrett, J. N., K. L. Magleby, and B. S. Pallotta. 1982. Properties of single calcium-activated potassium channels in cultured rat muscle. *J. Physiol.* 331:211-230.
- Blatz, A. L., and K. L. Magleby. 1986. Quantitative description of three modes of activity of fast chloride channels from rat skeletal muscle. *J. Physiol.* 378:141-174.
- Colquhoun, D., and A. G. Hawkes. 1977. Relaxation and fluctuation of membrane currents that flow through drug-operated channels. *Proc. R. Soc. Lond. B.* 199:231-262.
- Colquhoun, D., and A. G. Hawkes. 1981. On the stochastic properties of single ion channels. *Proc. R. Soc. Lond. B.* 211:205-235.
- Colquhoun, D., and A. G. Hawkes. 1982. On the stochastic properties of bursts of single ion channel openings and of clusters of bursts. *Phil. Trans. R. Soc. Lond. B.* 300:1-59.
- Colquhoun, D., and A. G. Hawkes. 1983. The principles of the stochastic interpretation of ion channel mechanisms. *Single Channel Recording*. B. Sakmann and E. Neher, editors. Plenum Press, New York. 135-175.
- Colquhoun, D., and A. G. Hawkes. 1987. A note on correlations in single ion channel records. *Proc. R. Soc. Lond. B.* 230:15-52.
- Colquhoun, D., and B. Sakmann. 1985. Fast events in single-channel currents activated by acetylcholine and its analogues at the frog muscle end-plate. *J. Physiol.* 369:501-557.
- Ferguson, W. B., O. B. McManus, and K. L. Magleby. 1993. Opening and closing transitions for BK channels often occur in two steps via sojourns through a brief lifetime subconductance state. *Biophys. J.* 65:702-714.
- Fredkin, D. R., M. Montal, and J. A. Rice. 1985. Identification of aggregated Markovian models: application to the nicotinic acetylcholine receptor. *In Proceedings of the Berkeley Conference in Honor of Jerzy Neyman and Jack Kiefer*. L. M. LeCam and R. A. Olshen, editors. Wadsworth Press, Belmont. 269-289.
- Hamill, O. P., A. Marty, E. Neher, B. Sakmann, and F. J. Sigworth. 1981. Improved patch clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch.* 391:85-100.
- Hamill, O. P., and B. Sakmann. 1981b. Multiple conductance states of single acetylcholine receptor channels in embryonic muscle cells. *Nature.* 294: 462-464.
- Hille, B. 1992. *Ionic Channels of Excitable Membranes*. Sinauer Associates, Sunderland, MA. 607 pp.
- Kerry, C. J., R. L. Ramsey, M. S. P. Sansom, and P. N. R. Usherwood. 1988. Glutamate receptor channel kinetics. The effect of glutamate concentration. *Biophys. J.* 53:39-52.
- Kijima, S., and H. Kijima. 1987. Statistical analysis of channel current from a membrane patch. I. Some stochastic properties of ion channels or molecular systems in equilibrium. *J. Theor. Biol.* 128:423-434.
- Kirber, M. T., J. J. Singer, J. V., Walsh, Jr., M. S. Fuller, and R. A. Peura. 1985. Possible forms for dwell-time histograms from single-channel current records. *J. Theor. Biol.* 116:111-126.
- Latorre, R., A. Oberhauser, P. Labarca, and O. Alvarez. 1989. Varieties of calcium-activated potassium channels. *Annu. Rev. Physiol.* 51:385-399.
- Lauger, P. 1983. Conformational transitions of ionic channels. *In Single Channel Recording*. B. Sakmann and E. Neher, editors. Plenum Press, New York. 177-189.
- Magleby, K. L., and D. S. Weiss. 1990a. Estimating kinetic parameters for single channels with simulation. A general method that resolves the missed event problem and accounts for noise. *Biophys. J.* 58:1411-1426.
- Magleby, K. L., and D. S. Weiss. 1990b. Identifying kinetic gating mechanisms for ion channels by using two-dimensional distributions of simulated dwell times. *Proc. R. Soc. Lond. B.* 241:220-228.
- Marty, A. 1981. Ca-dependent K channels with large unitary conductance in chromaffin cell membranes. *Nature (Lond.)*. 291:497-500.
- McManus, O. B., A. L. Blatz, and K. L. Magleby. 1985. Inverse relationship of the durations of adjacent open and shut intervals for Cl and K channels. *Nature.* 317:625-628.
- McManus, O. B., A. L. Blatz, and K. L. Magleby. 1987. Sampling, log binning, fitting, and plotting durations of open and shut intervals from single channels and the effects of noise. *Pflügers Arch.* 410: 530-553.
- McManus, O. B., and K. L. Magleby. 1988. Kinetic states and modes of single large-conductance calcium-activated potassium channels in cultured rat skeletal muscle. *J. Physiol.* 402:79-120.
- McManus, O. B., and K. L. Magleby. 1989. Kinetic time constants independent of previous single-channel activity suggest Markov gating for a

- large-conductance Ca-activated K channel. *J. Gen. Physiol.* 94: 1037-1070.
- McManus, O. B., and K. L. Magleby. 1991. Accounting for the Ca²⁺-dependent kinetics of single large-conductance Ca²⁺-activated K⁺ channels in rat skeletal muscle. *J. Physiol.* 443:739-777.
- Neher, E., and C. F. Stevens. 1977. Conductance fluctuations, and ionic pores in membranes. *Annu. Rev. Biophys. Bioeng.* 6:345-381.
- Pallotta, B. S., K. L. Magleby, and J. N. Barrett. 1981. Single channel recordings of Ca²⁺-activated K⁺ currents in rat muscle cell culture. *Nature (Lond.)* 293:471-474.
- Patlak, J. B. 1988. Sodium channel subconductance levels measured with a new variance-mean analysis. *J. Gen. Physiol.* 92:413-430.
- Rae, J. L., J. Dewey, J. S. Rae, and K. Cooper. 1990. A maxi calcium-activated potassium channel from chick lens epithelium. *Curr. Eye Res.* 9:847-861.
- Richard, E. A., and C. Miller. 1990. Steady-state coupling of ion-channel conformations to a transmembrane ion gradient. *Nature.* 247:1208-1210.
- Sigworth, F. J., and S. M. Sine. 1987. Data transformations for improved display and fitting of single-channel dwell time histograms. *Biophys. J.* 52:1047-1054.
- Snedecor, G. W., and W. G. Cochran. 1989. *Statistical Methods.* Iowa State University Press, Ames, IA. 76, 468.
- Steinberg, I. Z.. 1987a. Frequencies of paired open-closed durations of ion channel. *Biophys. J.* 52:47-55.
- Steinberg, I. Z. 1987b. Relationship between statistical properties of single ionic channel recordings and the thermodynamics state of the channels. *J. Theor. Biol.* 124:71-87.
- Tyerman, S. D., B. R. Terry, and G. P. Findlay. 1992. Multiple conductances in the large K⁺ channel from *Chara corallina* shown by a transient analysis method. *Biophys. J.* 61:736-749.