702 Biophysical Journal Volume 65 August 1993 702-714

Opening and Closing Transitions for BK Channels Often Occur in Two Steps via Sojourns through a Brief Lifetime Subconductance State

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ABSTRACT Single channel currents were recorded with microsecond time resolution from large-conductance calcium-activated K^+ channels to examine the details of the opening and closings transitions. Analysis of averaged closing transitions indicated that the initial average conductance step for closing was to the 90–95% closed channel current level. Averaged brief closings (~50 μs) reopened from the initial 90–95% level, whereas averaged longer closings (>300 μs) closed completely from this level over the next 50–100 μs. The 90–95% initial closed level in the averaged current records resulted typically from the average of both complete and partial closings. From 45–80% of the initial closings were complete and 20–55% were to brief lifetime (~50 μs) subconductance levels at 65–90% of the completely closed level. Averaged opening transitions were typically mirror images of averaged closing transitions. To extend the analysis to the very brief conductance changes that underlie the flickers of the single channel current toward the closed current level, flickers, brief closings, and longer closings were averaged separately and their slopes compared. The slopes were similar (within the 3% resolution of the method), suggesting similar initial conductance steps. Similar initial closing properties for both the briefer and longer closings would be expected if the channel first passed through the kinetic and subconductance states associated with the briefer closings (including flickers) before entering the longer closed states. Such transitions would provide an explanation for the observation that openings and closings often occur in two steps.

INTRODUCTION

In 1977 Colquhoun and Hawkes predicted that activation of a channel by an agonist should produce several openings in quick succession. Such bursts of activity were soon observed at the single channel level (Nelson and Sachs, 1979; Colquhoun and Sakmann, 1981; Barrett et al., 1982). While bursts of activity are now accepted as a characteristic feature of single channel recordings, the nature of the brief closings is unclear. Some of the brief closings are complete, others are to subconductance levels, and still others, often the majority, are so brief that they appear only as flickers of the open channel current toward the closed current level (Barrett et al., 1982; Auerbach and Sachs, 1983, 1984; Colquhoun and Sakmann, 1985; Tyerman et al., 1992). Flickers could arise from brief closings to the zero current level (complete closings) or from brief closings to subconductance levels (partial closings). As detailed by Colquhoun and Sigworth (1983), it would be difficult to distinguish between these two possibilities for any given flicker, due to the limited frequency response and inherent noise of single channel current records. Nevertheless, it is important to establish whether flickers, on average, arise from partial or complete closings, as different conductance levels suggest different closed conformations.

In this paper we examine whether flickers close to the same level as longer closings by comparing the slopes of averaged flickers to the slopes of averaged longer closings. Similar slopes (within the 3% resolution of the method) were found for flickers and longer closings, suggesting that, on average, flickers and longer closings close initially to similar conductance levels. An unexpected finding of this study was that the average initial conductance level for flickers, brief closings, and longer closings was not the completely closed level, but a subconductance level at 90–95% of the completely closed level. Averaged flickers and brief closings then reopened from this subconductance level, and averaged longer closings then closed completely from this subconductance level over the next 50–100 μs, giving rise to a creep in the current to the baseline after the initial rapid closing step. Averaged openings were typically mirror images of averaged closings, displaying a creep before the rapid opening step. These results raise the possibility that the kinetic state that underlies the flickers and brief closings (flicker state) may be associated with the brief duration subconductance levels. The two-step closings and openings may then result when transitions to and from the longer closed states include sojourns through the flicker state with its associated subconductance levels. A preliminary report of some of these findings has appeared (Ferguson et al. 1992).

METHODS

Preparation and recording

Single-channel currents flowing through large-conductance Ca^{2+} activated K^+ channels (Marty, 1981; Pallotta et al., 1981) in surface membranes of primary cultures of rat skeletal muscle were recorded with the patch clamp technique (Hamill et al., 1981). Recordings were made from excised "inside-out" patches of membrane, with the normal inner membrane surface of the patch exposed to solutions in a micro-chamber, allowing solution changes (Barrett et al., 1982). The experiments for the majority of data presented in

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Received for publication 9 November 1992 and in final form 19 April 1993.

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0006-3495/93/08/702/13 $2.00

the paper, including the data for all the figures, except Fig. 5, were collected over an 18-month period of time using a single patch clamp amplifier carefully adjusted and consistently checked to provide appropriate responses to square wave inputs. Earlier experiments extending back 6 years examined amplitude-duration plots (such as Fig. 5) and slope comparisons. The results from the earlier experiments were consistent with the more recent experiments and have been included. Analyzed single channel data have been recorded under a variety of experimental conditions. In some experiments EGTA (typically 1 mM) was used to buffer the free Ca^{2+} concentration (details in McManus and Magleby (1988)). TES buffer (*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid) 2–5 mM was used in all solutions to hold the pH at 7.0. Data were collected with either 140–150 (symmetrical) KCl on both sides of the membrane, or with asymmetric KCl solutions to increase the amplitudes of the single channel currents, as indicated in the figure legends, where "extracellular solution" refers to the solution in the patch pipette and "intracellular solution" refers to the solution at the inner membrane surface of the excised patch. While most data were collected at room temperature (21–24°C), some experiments were performed at lower temperatures (5–10°C).

The membrane potential of the excised patches was held at either +20 or +30 mV, as indicated in the figure legends. Membrane potential is expressed in the conventional manner for intact cells, with the potential indicating the voltage at the normal "intracellular" side of the membrane. Currents were stored on FM tape during the experiment with a high frequency cutoff of typically 37 kHz (Racal, Store 4DS). The tape speed was then slowed, typically 16–64 times and the current filtered further for sampling by computer. The effective high frequency cutoff when considering the filtering from all components for experiments with symmetrical KCl was typically in the range of 4–10 kHz (−3 dB, four-pole Bessel filtering). For experiments with high concentration gradients of K^+ and resulting large currents of 20–40 pA, the effective high frequency cutoff for the analysis was 15–25 kHz, depending on the experiment, as indicated in the figure legends.

Analysis

A number of methods have been developed for the analysis of subconductance levels (Auerbach and Sachs, 1983, 1984; Colquhoun and Sigworth, 1983; Colquhoun and Sakmann, 1985; Sigworth, 1986; Patlak, 1988; Tyerman et al., 1992). We have used and extended some of these approaches, and devised new ones as necessary, to meet the requirements of our study. Analysis was performed on LSI 11/73 computers (Digital Equipment Corporation, Marlboro MA) using DEC 11 BASIC modified with assembler language programs written in the laboratory. Slowing of the tape speed for analysis allowed effective sampling rates of 1–8 μs (typically 1.5–3 μs). The sampled data were then displayed, and closed intervals were selected automatically, based on the time the single channel current was above and below various threshold levels, as indicated in the figure legends.

The automatically selected intervals were then displayed to allow operator inspection and possible exclusion of selected intervals from the averaged displays. In some cases no intervals or only obvious artifacts (such as tape drop-outs) were excluded from the averaging. In others, intervals with obvious subconductance levels were either excluded or selected, as indicated in the Results. Averaged intervals were aligned based on the time of crossing of a threshold level, usually set at about the midpoint of the observed current transition being examined. Numerical values of the slopes were calculated from the change in current and time between sampled points. Accurate determination of slopes required that there be at least 10, and preferably 20, sampling points falling between the 10–90% open and closed current levels. Increased noise would be expected to increase error in estimation of slopes. For the experiments presented here the standard deviation of the current noise in the absence of channel activity was typically less than 4–10% of the single channel current amplitude.

In some experiments simulated single channel current records were analyzed to determine the resolution and possible artifacts associated with the methods of analysis. Simulated currents were obtained by first generating idealized single channel currents (assuming no noise and filtering) using models consistent with the kinetics of the BK channel (McManus and Magleby, 1991). These idealized currents were then filtered, and filtered noise obtained from a patch clamp amplifier or an experiment was added to give currents with filtering and noise equivalent to the filtering and noise in the experimental currents. In some of the simulated currents brief closures were to conductance levels other than zero. These simulated records were then analyzed in the same manner as used for the experimental current records. The percentage difference between slope A and slope B was calculated from: 100% (slope A − slope B)/slope A.

For each figure, plots with the same letter, such as B1 and B2, are from the same channel (experiment) or simulation, whereas plots with different letters, such as A and B, represent data from different channels or experiments.

RESULTS AND DISCUSSION

Flickers, closings to subconductance states, and complete closings

Fig. 1 shows single channel current records obtained from a large-conductance Ca^{2+}-activated K^+ (BK) channel in an excised patch of membrane from primary cultures of rat skeletal muscle. The wide range of durations of the various open and closed intervals reflect the complex kinetics of the gating process arising from transitions among three or more open and five or more closed states (McManus and Magleby, 1988, 1991). It is also apparent that all closed intervals do not reach the same current level. Examples of flickers (*F*), a closing to a subconductance level (*S*), and complete closings (*C*) are indicated. In contrast to both the complete closings and the closing to the subconductance level, flickers are of such brief duration that they do not reach a steady-state level, due to the limited frequency response of the single channel current record (Colquhoun and Sigworth, 1983).

Because flickers do not reach a steady-state level, it is not possible to determine by simple inspection of the current record whether they arise from complete or partial closings. The reasons for this are shown in Fig. 2, *A* and *B* (Colquhoun and Sigworth, 1983; Colquhoun and Sakmann, 1985). *A1* and

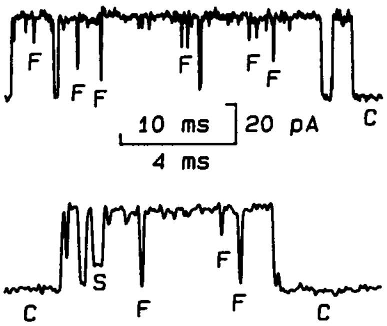

FIGURE 1 Currents recorded from a single large-conductance Ca^{2+}-activated K^+ channel in an excised membrane patch. Extracellular solution: 50 mM KCl and 5 mM TES; intracellular solution: 2 M KCl, 5 mM TES, and 10 μM added Ca^{2+}. Single channel current records were filtered with an effective high frequency cutoff of 4.9 kHz and sampled by computer with an effective sampling interval of 4.7 μs. The sampled data were then plotted by connecting the sampled points. The filtering was greater than used for analysis of the data (see Fig. 6*A*) so that the various types of closings would be clearly visible: *F*, flickers; *S*, a closing to a subconductance level; *C*, complete closings. Membrane potential, +20 mV.

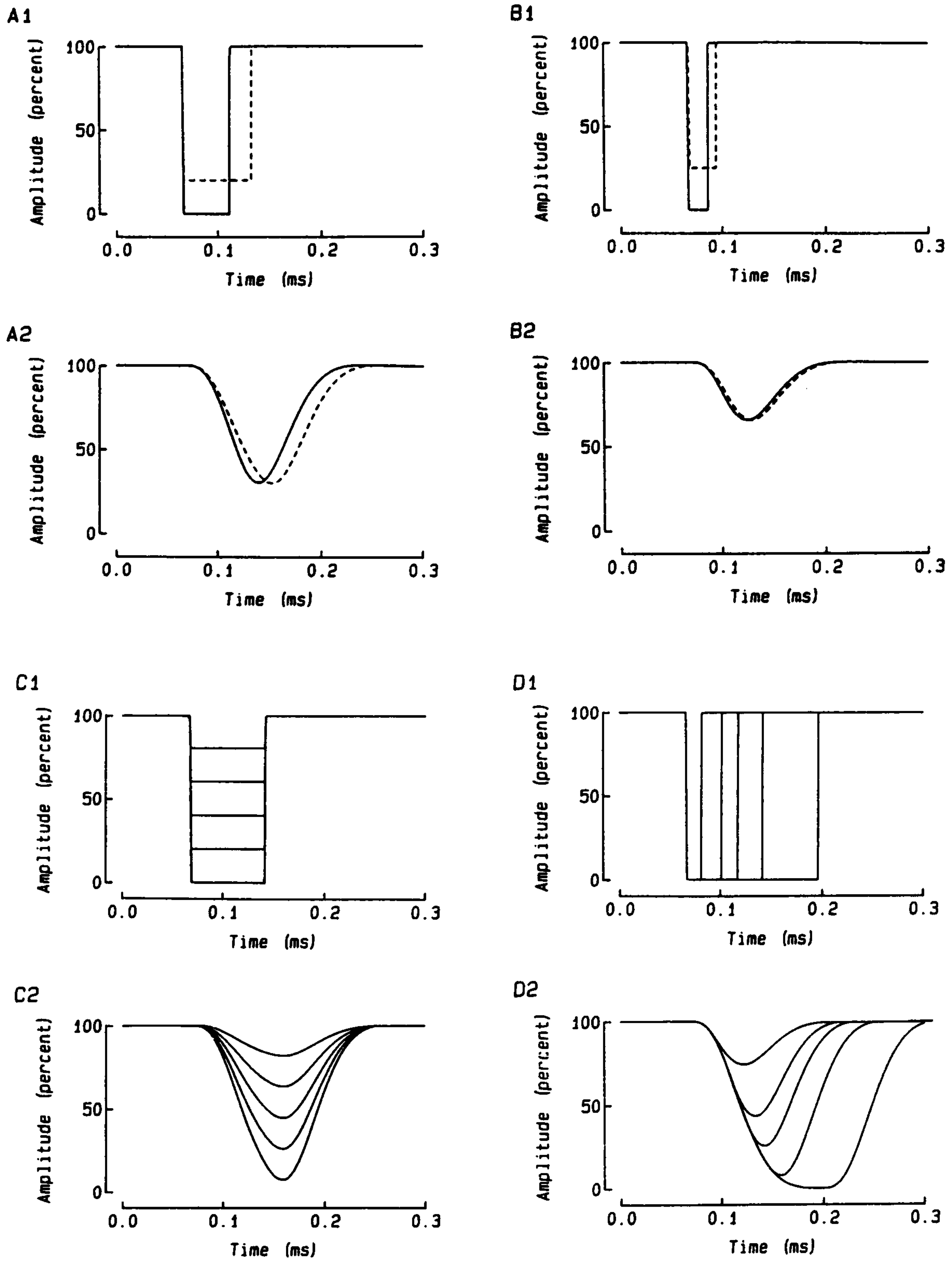

FIGURE 2 The slope of a filtered step response is proportional to the amplitude of the underlying current step. (*A1* and *B1*) Current steps of different amplitudes and durations. (*A2* and *B2*) Observed responses after filtering the current steps in *A1* and *B1*, respectively, at an effective high frequency cutoff of 6.2 kHz (four-pole approximate Bessel filter). (*C1* and *D1*) Current steps of constant duration and constant amplitude, respectively. (*C2* and *D2*) Filtered responses for the current steps in *C1* and *D1*, respectively. Note that the slopes of the filtered responses are proportional to the amplitudes of the current steps. The results can be scaled to any level of filtering by multiplying the time by: 6.2 kHz/(desired level of filtering in kHz). Thus, for filtering with a high frequency cutoff of 3.1, 12.4, or 24.8 kHz, each division on the time base becomes 0.2, 0.05, or 0.025 ms, respectively.

B1 present currents through hypothetical single channels that have instantaneous opening and closing transitions to both complete (*continuous lines*) and partial (*dashed line*) closed levels. *A2* and *B2* present the currents that would be observed with the level of filtering typically required to reduce the current noise in a patch clamp recording to acceptable levels. The durations of the complete and partial closings were selected to produce flickers that reached 70% (*A*) and 35% (*B*) of the closed current level. In both *A* and *B*, flickers of identical amplitudes were produced by either partial or complete closings. Thus, a characteristic other than amplitude must be used to determine whether flickers arise from complete or partial closings. In both *A* and *B*, the flicker arising from the complete closing (*continuous lines*) was found to have the steeper slope, suggesting that slope may provide a tool to distinguish complete from partial closings.

Initial slopes indicate the magnitude of the step closing

That slopes might be used to determine whether flickers arise from complete or partial closings is shown in Fig. 2, *C* and *D*, which present current steps and filtered responses for hypothetical channels. In *C2* the slope of the observed flicker was proportional to the amplitude of the current step for the constant duration closings shown in *C1*. In *D2* the slopes of the observed flickers were identical for the closings of different durations to the same (zero) current level shown in *D1*. Thus, a comparison of the slopes of flickers to the slopes of complete closings would indicate the closing level that produced the flicker. For example, if the slope of the flicker were 80% of the slope of a complete closing, then the flicker would arise from an 80% closing (a closing to a subconductance state with a conductance 20% of the open channel conductance). Simply stated, the slope is proportional to the magnitude of the current step, since the rate of voltage change across an active Bessel filter (or a capacitor for a simple filter) is proportional to the magnitude of the charging (or discharging) current.

Due to noise in experimental single channel current records (Fig. 1), it is not possible to make accurate estimates of slopes from a single event. However, averaging a sufficient number of flickers and longer duration (complete) closings should average out the noise so that comparisons in slope can be made. To test whether such an approach is applicable, simulated single channel current records with filtering and noise similar to experimental currents were generated and analyzed. For some simulated current records the brief events that gave rise to flickers were complete closings and in others they were partial closings to various conductance levels. A brief segment of a current generated by simulation is shown in Fig. 3 *A1*. The entire current record was then analyzed by sampling the simulated current record and storing the digitized amplitude in a large file. The file was then searched to find longer closings (which were complete) and flickers that just crossed the dashed line at the 65% closed level in Fig. 3 *A1*. (The flicker just before the 0.6-ms closing would have been selected from this record.) The selected flickers and complete closings were then averaged separately and displayed (see Methods).

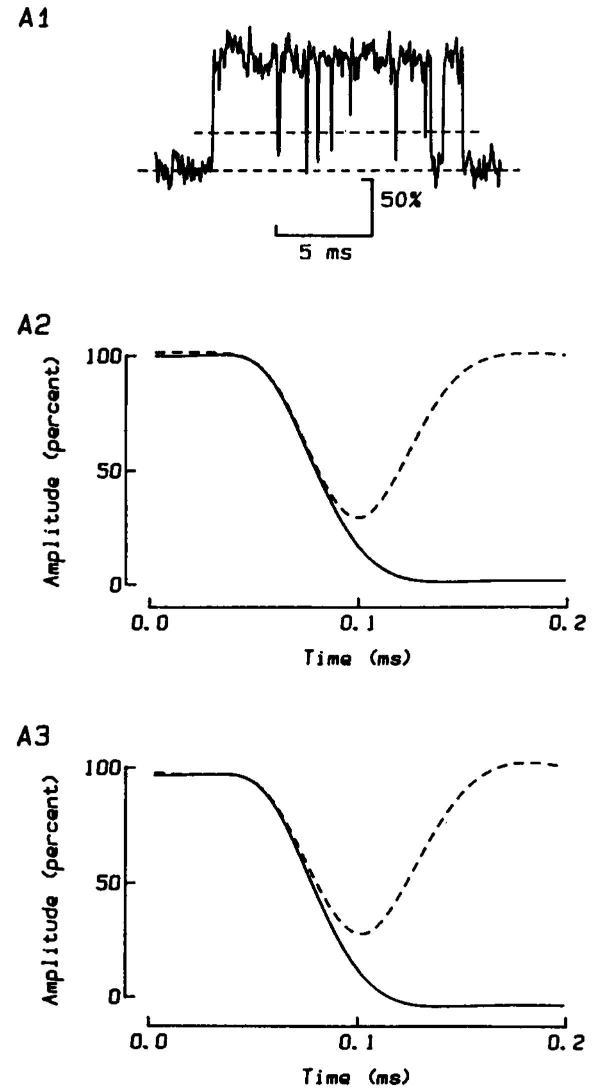

FIGURE 3 The slopes of the averaged flickers in simulated single channel currents are proportional to the amplitudes of the underlying current steps. (*A1*) A simulated single channel current with noise and filtering (effective filtering, 7 kHz; effective sampling interval, 3.5 μs). For this record the current steps underlying the briefest closures were 90% closures. (*A2*) Averaged flickers (*dashed line*) arising from complete (100%) closings in a simulated current with filtering and noise have a closing transition slope that is similar (2.2% less steep) to that of averaged longer complete closings (*continuous line*); 49 flickers and 166 longer openings were averaged. (*A3*) Averaged flickers (*dashed line*) arising from 90% closings have a transition slope 9.1% less than the slope for averaged longer complete closings (*continuous line*); 47 flickers and 187 longer closings were averaged. For both *A2* and *A3* flickers with one to seven sampling points below the 70% threshold line were averaged for comparison to longer duration (>695 μs) shut intervals.

Fig. 3 *A2* presents results from an analysis in which the flickers arose from complete closings. In this case the flickers (*dashed line*) had a slope similar to the longer complete closings (*continuous line*). Fig. 3 *A3* presents results in which the flickers arose from brief partial closings that were only 90%

complete. The reduced slope of the flickers arising from partial closings when compared to the slope of the longer complete closings is evident. Measurements indicated that the slope for the flickers was 9% less than the slope for complete closings, in close agreement with the expected reduction of 10%. Analysis of a series of simulated single channel current records of the type shown in Fig. 3 indicated that estimates of the current steps from the slopes for averaged data were typically within 3% of the current steps used to simulate the data, provided that the selected flickers reached the 40% or greater closed level. Thus, the error in estimating current steps from experimental data should also be within about 3% since the noise and kinetics of the experimental data are similar to those for the simulated data.

Flickers and longer closings have similar slopes

Fig. 4*A1* presents a current record from a single BK channel. One complete closing and six obvious flickers are apparent. To increase the open channel current, the recordings were obtained with 500 mM K^+ at the inner membrane surface and no added K^+ in the pipette (which contained 150 mM *N*-methyl-D-glucamine). The high signal to noise ratio resulting from the 18-pA currents allowed the current to be

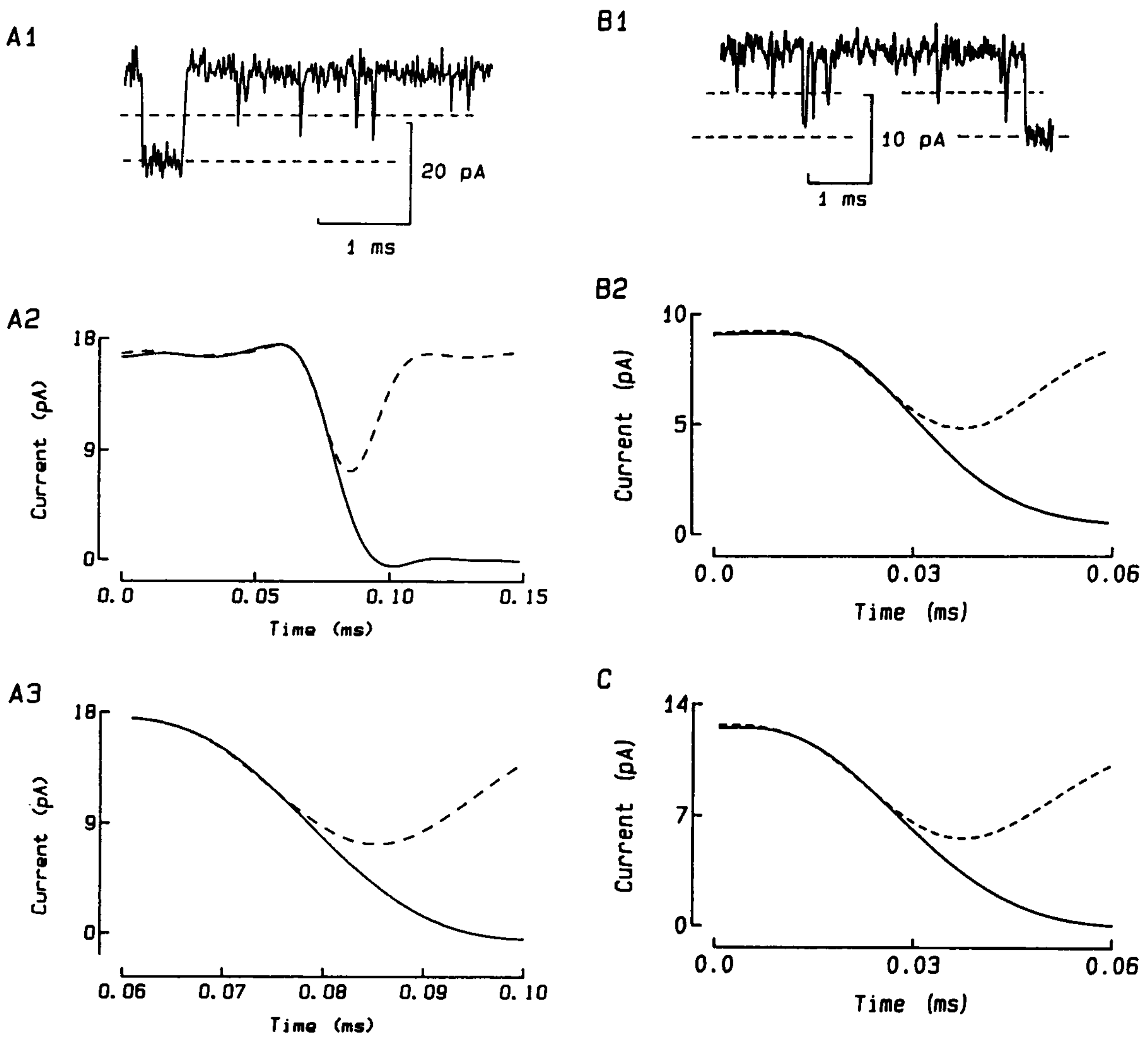

FIGURE 4 Flickers and longer closings from single large-conductance Ca^{2+}-activated K^+ channels have similar slopes. (*A1*) Single channel current record. (*A2* and *A3*) Averaged flickers (*dashed line*) have a closing transition slope that is similar (2% steeper) to that of longer (>78 μs) closings. *A3* plots the data in *A2* on a faster time base. Averaged flickers had one to five sampling points below the 50% threshold line: 689 flickers and 288 longer closings were averaged. Extracellular solution, 150 mM *N*-methyl-D-glucamine and 5 mM TES buffer; intracellular solution, 500 mM KCl, 5 mM TES, and 50 μM added CaCl. Filtering, 15.2 kHz; sampling period, 1.56 μs. Membrane potential, +30 mV. (*B1*) Current record from another channel. (*B2*) Averaged flickers (*dashed line*) have a closing transition slope that is similar (2.3% steeper) to that of longer (>156 μs) closings. Averaged flickers had one to three sampling points below a 35% (*closed*) threshold line, 366 flickers and 395 longer closings were averaged. Extracellular solution, 150 mM KCl, 5 mM TES, and 1 mM EGTA; intracellular solution, 150 mM KCl, 5 mM TES, and 50 μM added Ca^{2+}. Effective filtering, 13.2 kHz; effective sampling interval, 3.1 μs. Membrane potential, +30 mV. (*C*) Averaged flickers (*dashed line*) from another channel at 10°C have a closing transition slope that is similar (1.9% less steep) to that of longer (>78 μs) closings. Averaged flickers had one to five sampling points below a 50% threshold line. Extracellular solution, 150 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM TES; intracellular solution, 500 mM KCl, 50 μM added Ca^{2+}, 5 mM TES. Effective filtering, 11.1 kHz; effective sampling interval, 1.56 μs. Average response of 61 flickers and 128 longer closings. Membrane potential, +30 mV.

analyzed with high time resolution (effective cut-off frequency of 15.2 kHz). Detected flickers just crossing the dashed line at the 50% level (illustrated in Fig. 4 *A1*) were then averaged for comparison to longer (complete) closings from the same current record. From Fig. 4, *A2* and *A3*, it can be seen that the averaged flickers and averaged complete closings had similar slopes (within 2%). Indistinguishable slopes (within the 3% resolution of the method) suggest that the magnitude of the initial conductance step for the average flicker is the same as the magnitude of the initial conductance step for longer closings. Thus, both flickers and longer closings close initially to the same average current level.

It can be calculated (Colquhoun and Sigworth, 1983) that the brief closings that gave rise to the average flicker in Fig. 4 *A* had an average duration of about 11 μs. Thus, the initial closed current level for closings as brief as 11 μs was similar to the initial closed current level for longer closings. (The increase in the open current before the closing transition in Fig. 4 *A2* arises from the averaging of currents from channels that are open for different durations before the closing. This effect will be more pronounced in some of the later figures.)

Fig. 4, *B1* and *B2*, present results from a different membrane patch bathed in symmetrical 150 mM KCl, and Fig. 4 *C* presents results obtained at low temperature (10°C) from another membrane patch with 150 mM KCl in the pipette and 500 mM KCl at the inner membrane surface. In both cases the slopes of the flickers and longer (complete) closings were similar.

For over 30 plots of the type shown in Fig. 4 for flickers that reached from 40 to 70% of the closed current amplitude, depending on the experiment, flickers and longer closings had similar slopes for the seven examined BK channels, and this was the case with symmetrical 150 mM KCl and also with various K^+ gradients to give up to 40-pA currents, so that flickers arising from closings as brief as 7 μs could be measured. Similar slopes suggest that flickers and longer closings have similar initial conductance steps.

Individual closings with durations greater than 100–200 μs typically reach the zero current level

The results in Fig. 4 indicated that the initial conductance step for the very brief closings underlying flickers was not measurably different from the initial conductance step for longer closings. The results did not indicate, however, whether the initial conductance steps for the longer closings used for comparison were complete. Consequently, we examined both individual longer closings and averaged longer closings to determine if they were complete closings. To examine individual longer closings, the steady-state current amplitudes and durations of the closings were measured with operator adjusted cursor lines from visual displays of current records and plotted at two time resolutions in Fig. 5, *A1* and *A2*. The 10-pA level in the plot represents the open channel current and the 7.3-kHz high-frequency cut-off allowed closings with durations >100 μs to reach a steady-state level and be measured directly.

To control for the effects of noise, filtering, and channel kinetics on the measured amplitudes, single channel currents with noise and filtering were generated by simulation using a kinetic scheme that could describe the single channel kinetics of the measured current record (channel 5 in McManus and Magleby (1991)). The simulated currents were then measured and the results plotted in Fig. 5, *B1* and *B2*. Although there is more scatter in the experimental data (*A1* and *A2*) than in the simulated data (*B1* and *B2*), both plots have a similar numbers of observed closings to current levels other than zero. Since all closings used to generate the simulated data were complete closings, then the observed deviations from the zero current level for the simulated data must arise from the effects of noise and filtering on the simulated channel currents. Similar effects of noise and filtering would be expected for experimental channel currents. Thus, the similarity in the simulated and experimental data suggest that most closed events with durations greater than the 100-μs

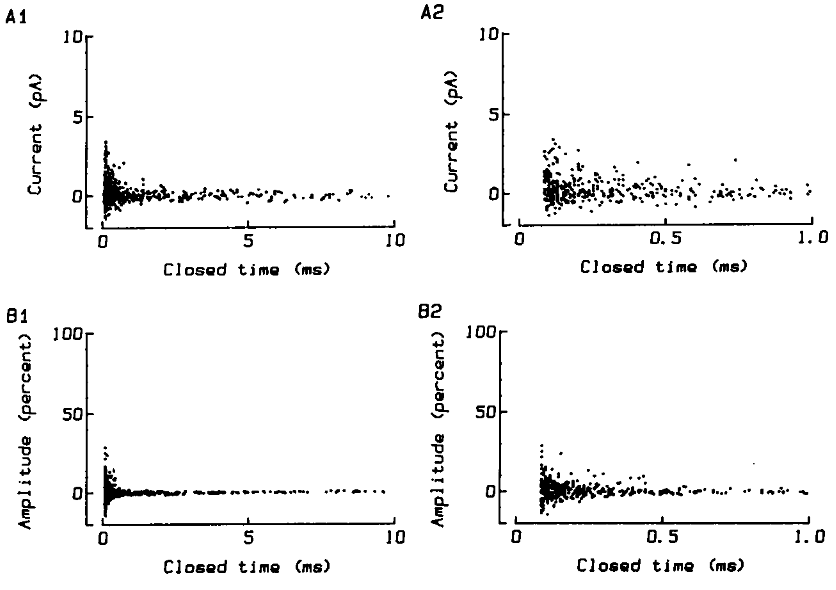

FIGURE 5 Closings longer than 100 μs are typically complete closings. Plots of the current amplitudes of single closings against their durations for experimental (*A1* and *A2*) and simulated (*B1* and *B2*) single channel current records with similar amounts of filtering and noise. Each point plots results from a single closing measured with cursors from a display. All closings used to generate the simulated current record were complete closings. Effective filtering, 7.3 kHz. Extracellular solution, 144 mM KCl, 2 mM TES, and 1 mM EGTA; intracellular solution, same as extracellular plus added Ca^{2+} to bring the free Ca^{2+} to 9.6 μM, as determined by method of Bers (1982). Membrane potential, +30 mV.

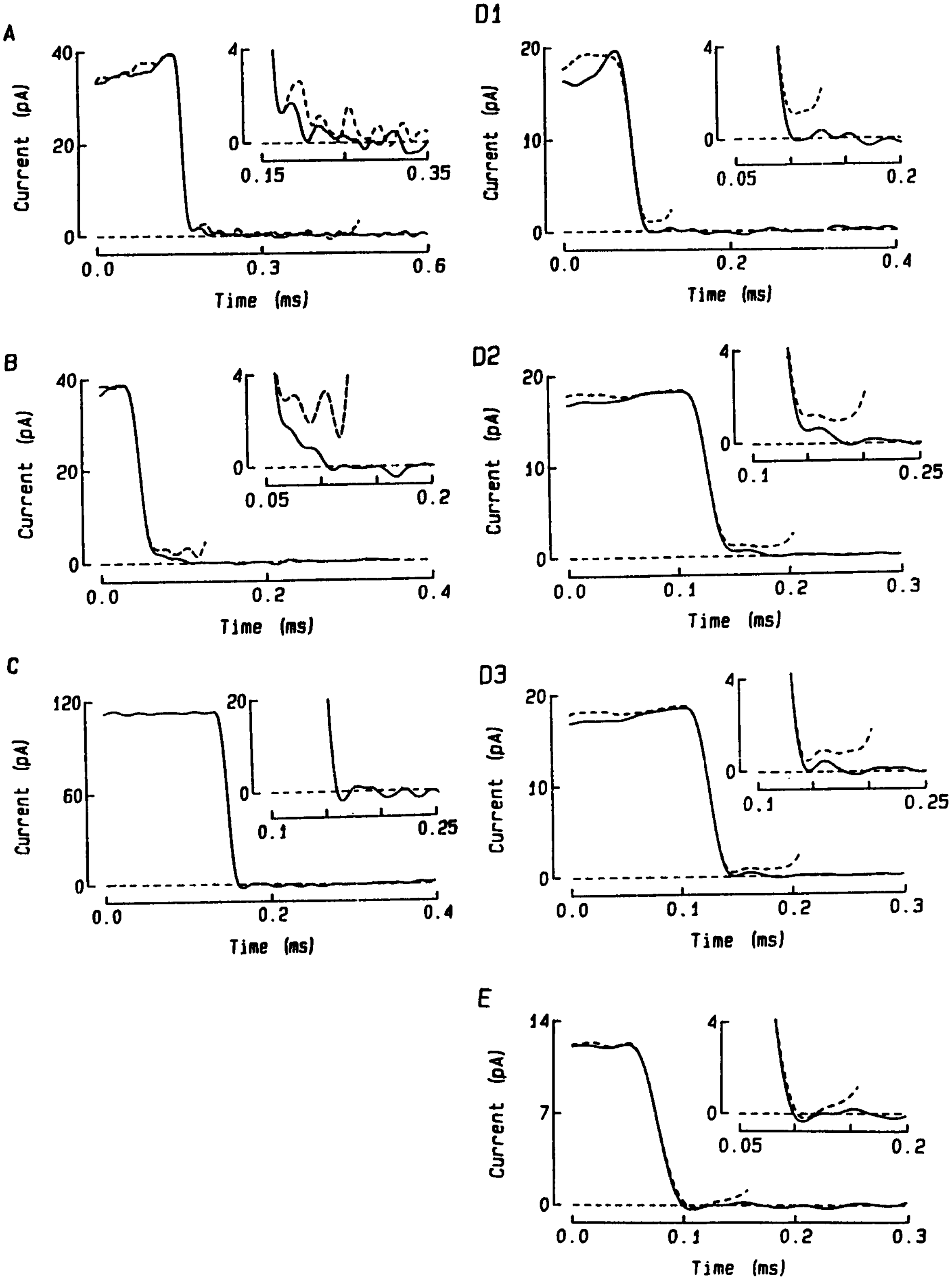

FIGURE 6 Averaged complete closings first close to 90–95% of the closed current level and then close completely over the next 50–100 μs. (*A*) Averaged single channel currents from intermediate duration closings (312–625 μs, *dashed line*) and longer duration closings (>625 μs, *continuous line*). The insert plots the data at increased resolution. Same experiment as Fig. 1, but with effective filtering of 22 kHz and effective sampling interval of 1.56 μs. Slope of intermediate duration closings is similar (1.6% steeper) to the slope of longer duration closings. (*B*) Averaged single channel currents from closings of brief durations (78–156 μs, *dashed line*) and longer durations (>312 μs, *continuous line*) have similar slopes and step to similar initial current levels. The slope of the brief closing is similar (2.6% steeper) to the slope of the longer closing. Extracellular solution, 20 mM KCl and 5 mM TES; intracellular solution, 1 M KCl, 5 mM TES, and 50 μM added Ca^{2+}. Effective filtering, 20.3 kHz; effective sampling interval, 1.56 μs. Membrane potential, + 30 mV. Records with obvious transitions through subconductance levels were excluded. (*C* and *D1–D2*) The creep after the initial closing step is not due to an artifact in

resolution of the analysis are complete closings. These findings can be compared to those of Auerbach and Sachs (1983) for the acetylcholine receptor in embryonic chick skeletal muscle where closings with durations of 250 μs (the briefest measured) to about 1.5 ms were not usually complete closings, but were typically to a 90% closed level.

Averaged longer closings first close to 90–95% of the completely closed level and then close completely over the next 50–100 μs

The advantage of plots like those shown in Fig. 5 *A* is that they display the current levels of individually measured closings. A disadvantage is that the usefulness of the measurements for estimating the underlying closed current levels is greatly reduced for closings with durations less than about 300 μs, due to the effects of filtering and noise. This is shown by the scatter in the measured current levels for brief closings in the simulated data in Fig. 5 *B*. To overcome the difficulties associated with the effects of noise and filtering on estimating the current levels of intermediate and brief duration single closings, we averaged closings with durations long enough to reach a steady state level. Closings of different durations were averaged separately. The slopes and closed levels of the different duration closings were then compared. In this way, closings with durations longer than the flickers examined in Fig. 4 and shorter than the longer closings examined in Fig. 5 could be evaluated. Results from an experiment of this type are shown in Fig. 6 *A*, where averaged closings of intermediate duration (312–624 μs) can be compared to averaged closings of longer duration (>624 μs). The intermediate duration closings (*dashed lines*) reached the same completely closed level as the longer closings (*continuous lines*), and the closing transitions for the intermediate and longer closings superimposed, yielding indistinguishable slopes (within 1.6%). Thus, except for duration, intermediate duration closings had similar closing properties as the longer duration closings.

A typical feature of both the intermediate and the longer closings in experiments of this type, was that the averaged current typically fell rapidly to a level that was about 90–95% of the completely closed level, and then fell more slowly over the next 50–100 μs to the fully closed level. An example from another membrane patch is shown by the continuous lines in Fig. 6 *B*. The creep could also be smaller, or absent, depending on the experiment and how the data were selected for analysis, as will be discussed in a later section.

Analysis of square wave currents and Ba^{2+} block indicates that the creep during longer closings is not an artifact of the recording and analysis system

A possible explanation for the creep in the closings shown in Fig. 6, *A* and *B* is that it arises in some manner from an artifact in the recording and analysis of the data. To investigate this possibility, square wave currents were introduced into the patch clamp amplifier, and the resulting output was analyzed in the exact same manner (including averaging) as used to analyze currents from single channels. Typical results are shown in Fig. 6 *C*. Following the step transition, the averaged current from the square wave fell below the baseline slightly, and then returned to the closed current level, just as would be expected from the small amount of ringing associated with Bessel filtering of a square wave; no creep was evident.

As a further test of whether the creep arose from an artifact, currents were recorded from a membrane patch with 50 μM Ca^{2+} added to the solution at the inner membrane surface or with both 50 μM Ca^{2+} and 0.5 mM Ba^{2+} added. As detailed previously (Vergara and Latorre, 1983; Miller, 1987; Neyton and Miller, 1988), the BK channel was almost continuously open with the high Ca^{2+}; adding 0.5 mM Ba^{2+} then led to the appearance of longer closed intervals. The averaged longer closed intervals, which presumably arose from Ba^{2+} block, since they were seldom observed in the absence of added Ba^{2+}, appeared as complete closings without creep (*continuous lines*, Fig. 6 *D1*), similar to the response obtained from the square wave current immediately after the experiment (Fig. 6 *C*).

The same channel which had no creep with presumed Ba^{2+} block showed creep in the absence of Ba^{2+} (*continuous lines*, Fig. 6 *D2*). Thus, the creep was not an obligatory artifact arising from the analysis, since it was not present with Ba^{2+} block or from analysis of square wave current steps. Furthermore, the creep in the experimental current records is probably underestimated, since the slight ringing of the Bessel filter would act to decrease the creep. The detection

the analysis. Averaged currents from a square wave introduced into the patch clamp amplifier (*C*) or from averaged longer closings arising mainly from Ba^{2+} block (*D1, continuous line*, see text) do not show creep when recorded and analyzed in the same manner as for the data in *D2* in the absence of Ba^{2+} block where creep is apparent. *D1* presents averaged single channel currents from closings with brief durations of 50–100 μs (*dashed lines*, average of 85 events) and longer durations > 530 μs presumably due to Ba^{2+} block (*continuous lines*, average of 53 events). Same experimental conditions as Fig. 4 *A*, except that 0.5 mM Ba^{2+} was added to the intracellular solution. (*D2* and *D3*) Averaged single channel currents for closings with brief durations of 75–150-μs (*dashed lines*) and longer durations >312 μs (*continuous lines*) in the absence of Ba^{2+}. Averages of 542 brief closings and 172 longer closings are presented in *D2*; the slope of the brief closings was 4% less than the longer closings. Notice the creep in *D2* (continuous line) in the absence of Ba^{2+} block. The data averaged for *D2* were then selected to exclude transitions through possible subconductance levels giving the results shown in *D3*, which presents averages of the 381 brief closings and 138 longer closings remaining after selection; the slope of the brief closing was 3% less than the slope of the longer closing. Same experimental solutions and membrane potential as Fig. 4 *A*. (*E*) Averaged single channel currents from brief closings with durations of 78–232 μs (*dashed lines*) and closings with longer durations > 234 μs (*continuous lines*). Records were selected to exclude apparent transitions through subconductance levels. There were 119 brief and 81 longer closings before selection and 55 brief and 70 longer closings after selection. 10°C. Same channel and experimental conditions as Fig. 4 *C*.

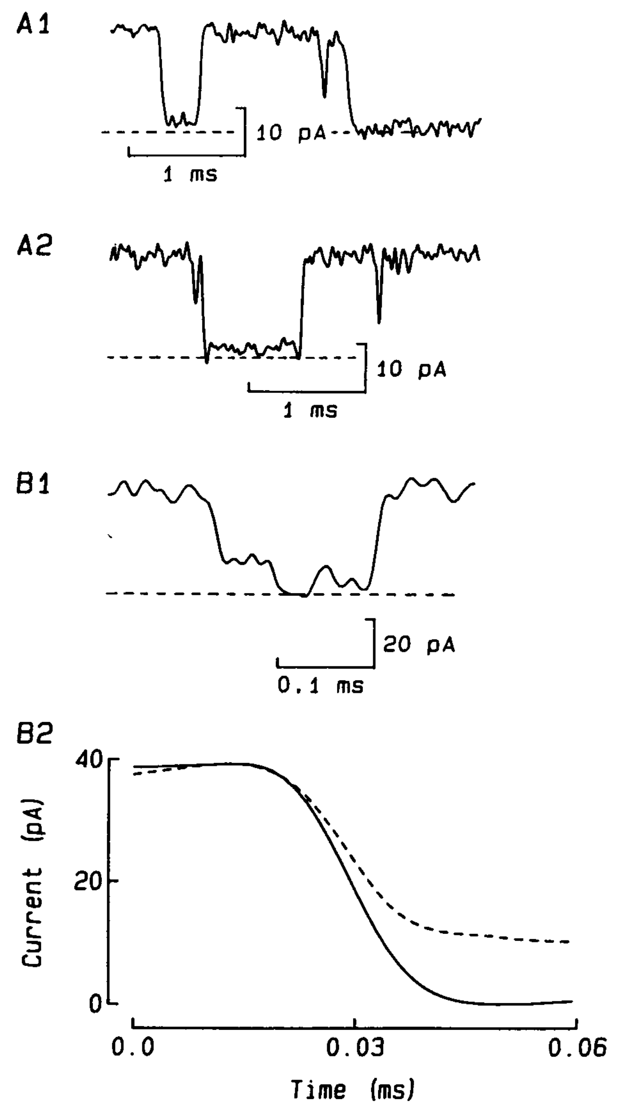

FIGURE 7 Transitions to subconductance levels. (*A1*, *A2*, and *B1*) Single channel currents showing apparent transitions to subconductance levels. The dashed lines indicate the closed channel current levels. For *A1* and *A2* the experimental conditions were the same as Fig. 6 *D*, but without Ba^{2+}. (*B2*) Averaged closings to a subconductance level (*dashed line*) at about the 70–75% closed level have a slope that is about 74% of the slope for complete closings (*continuous lines*). Records with transitions to the subconductance level shown in *B2* were selected from the complete current record and averaged for comparison to longer closings. For *B1* and *B2* the channel and experimental conditions were the same as Fig. 6 *A*.

of the creep in the experimental data required high time resolution (high frequency cut-off greater than 8 kHz) because of its brief duration.

Averaged brief closings are to the 90–95% closed level and have slopes similar to longer closings

The creep associated with the longer closings in Fig. 6, *A*, *B*, and *D2*, suggests that, if the channel reopened before it closed completely, then averaged closings with durations less than the duration of the creep (<100 μs) may not be complete. To test this possibility, brief closings with durations (below the 50% threshold level) of 78–156 μs were averaged for comparison to longer closings (> 312 μs). A minimum duration of 78 μs for a brief closing assures that the current would reach the completely closed current level if the closing were complete. This is the case since only about 30 μs would be required for the current to reach a steady-state level after a current step. The averaged brief closings (*dashed lines*) closed to about 90–95% of the completely closed level, before reopening, as shown by the dashed lines in Figs. 6, *B* and *D2*. Comparison of the slopes of the closing transitions indicated that the slopes of the averaged brief closings were indistinguishable (within the 3% resolution of the method) from the slopes of the averaged longer closings.

Thus, both averaged brief and averaged longer closings first typically closed to an apparent subconductance level that was about 90–95% of the completely closed level. The averaged brief closings then reopened from the subconductance level and the averaged longer closings then closed to the completely closed level over the next 50–100 μs. Similar results were obtained in about 80% of the 20 plots of this type for data obtained from seven channels for brief closings with durations in the examined range of about 50–200 μs. In the other 20% of the plots there was little or no apparent creep in the longer closings, and the brief closings approached the zero current level (not shown). Thus, in some cases most brief and longer closings step directly to the completely closed level. The reason for the variability among different patches was not apparent, but some variability might be expected since individual channels can possess differences in calcium sensitivity and kinetics (McManus and Magleby, 1991).

The creep in the averaged records is due mainly to step closures to subconductance levels

Averaged brief closings that close to the 90–95% closed level, as in Fig. 6, *B* and *D2*, could arise if all of the averaged closings were to subconductance levels, or if some of the closings were to subconductance levels and others were complete closings. To examine these possibilities, individual current records were visually inspected to identify closings to subconductance levels. Longer duration closures (>1–2 ms) to subconductance levels typically occurred so infrequently, about once per 1000 openings (Barrett et al., 1982), that they would have had little effect on the results, even if they had not been typically excluded before averaging. Examination of the records at higher time resolution indicated, however, that there were frequent closings of much briefer duration (<50–200 μs) to various subconductance levels. Examples are shown in Fig. 7, *A1* and *B1*. In some cases the subconductance levels were at about 10% of the open level (*A1*), and in other cases they were at about 25–35% (*B1*). The frequencies of apparent closings to the brief duration subconductance levels varied from as low as one per 10–100 closings to as high as one per two to three closings, depending on the particular single channel patch and temperature, with higher frequencies often occurring at lower temperatures (5–10°C versus room temperature). Lower temperatures are known to increase the frequency of transitions to subconductance levels (Tyerman et al., 1992).

Excluding obvious transitions to subconductance levels for the data averaged for Fig. 6 *D2* gave the results shown in Fig. 6 *D3*. The averaged longer closings (*continuous line*) now closed directly to the completely closed level and the averaged brief closings (*dashed line*) were almost complete, to within about 2% of the completely closed level.

As was the case before exclusion, the slopes of the brief and longer closings were typically similar (usually within < 4% in experiments of this type and within < 1% in a high resolution experiment with 40-pA currents). In selecting the data for Fig. 6 *D3*, about 30% of the brief closings and 20% of the longer closings were excluded. Thus, an observation of complete averaged closings after excluding 20–30% of the individual closings suggests that about 70–80% of the closings were directly to the completely closed level in this experiment.

Another example of complete closings for averaged data after excluding only a fraction of the individual events is shown in Fig. 6 *E*. Both averaged brief and averaged longer closings closed directly to the same completely closed level after excluding 55% of the brief closings and 15% of the longer closings (Fig. 6 *F*), suggesting that about 45–75% of the closings were directly to the completely closed level in this experiment performed at 10°C.

In some experiments (room temperature), averaged closings did not close directly to the completely closed level even after excluding apparent closings to subconductance levels. (The plot in Fig. 6 *B* shows an experiment of this type.) This could occur if the subconductance levels in the individual records were less than about 10–15% and too small to be consistently selected for exclusion, or if the conductance actually crept down in these experiments, rather than going through well defined subconductance levels.

The subconductance closings and creep in the averaged longer closings for the BK channel appear similar to the subconductance closings and creep observed by Auerbach and Sachs (1983, 1984) for acetylcholine receptor channels in cultured chick muscle, except for the much briefer time course for the BK channel. The ~50-μs mean lifetime of the apparent class of brief subconductance closings for the BK channel is about 10–20-fold briefer than the 0.5–1-ms mean lifetime of the class of subconductance closings in acetylcholine receptors (both estimates at room temperature).

Direct closures to subconductance levels

Transitions to subconductance levels might result from closings directly to the subconductance level, or alternatively, the channel might first close completely and then switch to a subconductance level, as shown in Fig. 7 *A2*. Although the switching is apparent in Fig. 7 *A2*, if the time spent in the full closure before switching would have been slightly less, then it would have appeared that the closing was directly to the subconductance level. To distinguish between closings directly to the subconductance level or complete closings with partial reopenings, closings to subconductance levels like those in Fig. 7 *B1* were averaged for comparison to complete closings. Of the 457 closings in the examined stretch of data, the 25 closings to a subconductance level of about 25–35% of the open channel conductance were selected for averaging. Results are shown in Fig. 7 *B2*. The slope of the averaged closing to the subconductance level was 26% less than the slope of the averaged complete closings. This is consistent with what would be expected if most of the subconductance closings in this experiment were directly to the subconductance level, and not rapid reopenings from complete closures.

Although the subconductance levels appear obvious in Fig. 7, it has not been ruled out that they arise from the time average of very rapid transitions between the open and closed current levels (e.g., Blatz and Magleby (1986)). It is also difficult to rule out the possibility that the subconductance levels and creep in the averaged data result from the averaging of such brief openings that they cannot be identified for exclusion in the individual records. For example, the lower current record in Fig. 1 has an apparent brief opening after the last closing. If this opening were briefer still, it might appear as a subconductance level.

Opening and closing transitions are typically symmetrical

In most experiments in which all opening and closing transitions were averaged without exclusion of obvious subconductance transitions, a creep similar to that observed in the longer closings also preceded the openings, as shown in Fig. 8 *A1*, such that the openings and closings were approximate mirror images of one another (Fig. 8 *A2*). Such symmetry in opening and closing transitions would be expected if the gating kinetics were consistent with microscopic reversibility, so that the numbers of transitions between any two given states would be the same in the forward and backward direction (e.g., Colquhoun and Hawkes (1983)). With microscopic reversibility, there is no net energy input into the gating process.

Conclusions and speculation

A major objective of this study was to determine whether the flickers in single channel currents in BK channels arise from complete or partial closings. Our results suggest that flickers (durations < 50 μs) arise, on average, from conductance steps to the 90–95% closed level. Interestingly, we also found that the initial closing transition for averaged brief closings (50–200 μs) and averaged longer closings (>300 μs) was also typically to the same apparent 90–95% closed current level. Flickers and brief closings then reopened, on average, from the 90–95% closed level, and longer closings then closed completely, on average, from the 90–95% closed level over the next 50–100 μs, producing a creep in the averaged currents to the zero current level.

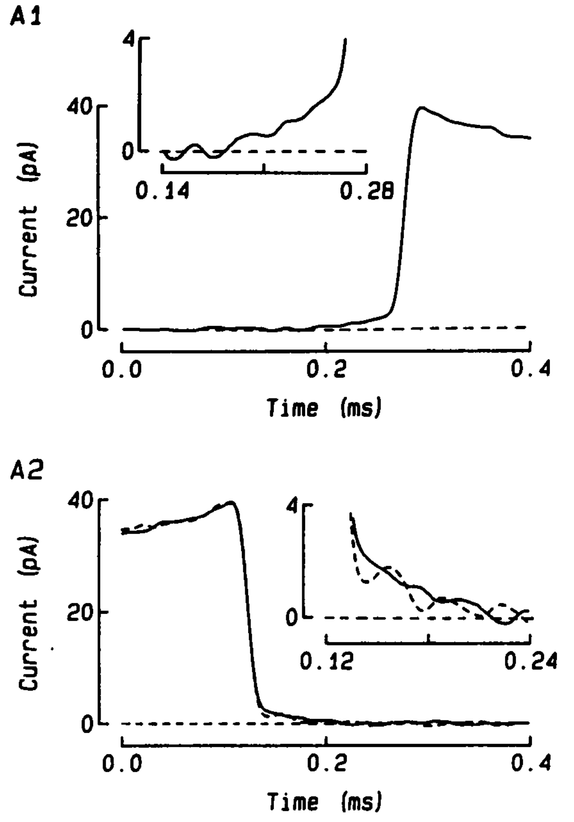

FIGURE 8 Averaged openings are typically mirror images of averaged closings. (*A1–A2*) Creep before opening (*continuous lines*) superimposes the creep after closing (*dashed lines*); the averaged opening is plotted with reverse time in A2. Opening transitions following and closing transitions preceding closed intervals >312 μs were averaged separately (averages of ~185 each). The opening and closing transition slopes were within 1%. Same experimental conditions as Fig. 6 *A*.

Indistinguishable average conductance steps (within the 3% resolution of the method) for flickers, brief closings, and longer closings are consistent with the possibility that the initial conformational changes that directly decrease channel conductance for all these events are, on average, typically the same. The high time resolution of the recordings extends this conclusion to closings as brief as 7 μs. Even if the initial conformational changes for the various closings are typically the same, the large differences in durations between the various closings, and the time-dependent decrease in the average conductance for the longer closings suggests that additional states are entered for the longer closings, consistent with conclusions from kinetic studies (McManus and Magleby, 1991).

Similar to the 50–100-μs creep following averaged longer closings, averaged openings were preceded by a creep, so that, in general, opening and closing kinetics were symmetrical in time. Thus, for longer closings, the channel first initially closed, on average, to the 90–95% closed subconductance level, followed by a closing to the completely closed level. This process was reversed on opening, with, on average, an initial opening to the 90–95% closed level, followed by a transition to the fully open level. The 90–95% closed level reached in the average initial closing transition arose typically from the average of closings directly to the fully closed level and closings to subconductance levels. About 45–80% of the closings were directly to the completely closed level, and about 20–55% were to subconductance levels. The subconductance levels were at about 65–90% of the completely closed level and the mean duration of time spent in the subconductance levels was about 50 μs. Thus, about 20–55% of the closings with durations longer than about 50 μs occurred in two steps. In these cases, the channel first closed to a 65–90% closed level for about 50 μs and then closed completely. A similar percentage of the openings also occurred in two steps through a reversal of the process.

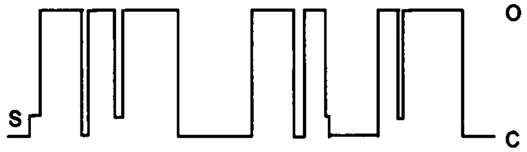

FIGURE 9 An idealized single channel current record for Scheme I. Opening and closing transitions can occur in two steps through a subconductance level with a mean lifetime of about 50 μs. Some of the very brief closings (flickers) reopen from the brief lifetime subconductance level without closing completely.

In a few experiments, most of the closings appeared complete, and in some other experiments it was not possible to exclude the possibility that the creep observed in the averaged data arose from a slowly changing current in the individual current records.

Subconductance levels of longer durations, of the types described previously (Barrett et al., 1982; Rae et al., 1990) occurred much less frequently than the subconductance levels of brief duration described above. The brief duration subconductance levels escaped detection in our previous studies because they were too brief to be apparent in the slower time bases typically used to examine the current records.

A simple model that summarizes our major observations is indicated by Scheme I, where the conformational states of the channel associated with the completely closed (C), subconductance (S), and open states (O) are indicated.

$$\begin{tikzcd} C \arrow[rr, shift left] \arrow[dr, shift left] & & O \arrow[ll, shift left] \arrow[dl, shift left] \\ & S \arrow[ul, shift left] \arrow[ur, shift left] & \end{tikzcd} \qquad (1)$$

Transitions can occur directly between the open and closed states or by passing through the subconductance states S. An idealized single channel current record for some typical transitions between the various conductance states is shown in Fig. 9.

A minimal kinetic gating mechanism for the BK channel is considerably more complex than Scheme I, as shown in Scheme II, where C and O represent closed and open kinetic states (McManus and Magleby, 1991).

$$\begin{array}{ccccccccc} C_8 & \rightleftharpoons & C_7 & \rightleftharpoons & C_6 & \rightleftharpoons & C_5 & \rightleftharpoons & C_4 \\ & & \upharpoonleft\downharpoonright & & \upharpoonleft\downharpoonright & & \upharpoonleft\downharpoonright & & \\ & & O_3 & \rightleftharpoons & O_2 & \rightleftharpoons & O_1 & & \end{array} \qquad (2)$$

The relationship between the conductance states in Scheme I and the kinetic states in Scheme II is not clear, but it may be useful to speculate on this question for the purpose of designing further experiments. Although all the states in Scheme II are usually entered during channel activity, the typical gating sequence for moderate levels of activity involves transitions mainly within the compound state described by: C_8-C_7-C_6-C_5-O_2-O_1 (McManus and Magleby, 1991). The closed state C_5 has a mean dwell time of about 50 μs, and the other closed states have considerably longer dwell times. Thus, in terms of Scheme II, most flickers and brief closed intervals result from transitions to C_5 from the compound open state O_1-O_2, such as: O_1-O_2-C_5-O_2-O_1 (McManus and Magleby, 1991).

If it is assumed that the conductances of the closed states C_8-C_7-C_6 are zero, that the conductances of the open states O_1 and O_2 are 100%, and that the conductance of the "closed" kinetic state C_5 is, on average, 5–10% of the open channel conductance, then openings would, on average, be preceded by a conductance step to the 5–10% open level as the channel passed through C_5 on its way from the longer closed states to the open states, such as: C_8-C_7-C_6-C_5-O_2-O_1. Conversely, the initial closings would, on average, be to the 90–95% closed level as the channel makes transitions from O_2 to C_5. Reopenings from C_5 would then give rise to flickers and brief closings with, on average, a 90–95% closed level associated with C_5. Transitions such as: O_1-O_2-C_5-C_6-C_7-C_8, from the open states through C_5 to one or more of the longer closed states would result in complete closings preceded by a creep in the averaged data, as the channel passed through C_5 to the completely closed states.

The rather limited support for this mechanism for brief subconductance levels comes from the observation that the mean dwell time in C_5 of about 50 μs estimated from kinetic studies (McManus and Magleby, 1991) is similar to the mean duration of the creep preceding channel opening and following channel closing. The kinetic state C_5 would represent a family of conformational states that have similar mean lifetimes of about 50 μs, but different conductances that range from zero to about 35% of the open channel conductance. A family of conformational states for C_5 could account for the observation of multiple subconductance levels of similar brief duration and also for the observation that the channel could also open and close without passing through obvious subconductance levels. Possible differences in BK channels (McManus and Magleby, 1991) and differences in experimental conditions could favor some conformations over others, giving rise to the observed variability in results.

It is also possible that the conformational changes that produce the brief duration subconductance levels are independent from those that produce the kinetic state C_5; the two processes may just happen to have similar lifetimes and act in parallel, giving a false appearance of coupling.

Further examination of the relationship, if any, between the kinetic state C_5 and the brief subconductance levels could be made by detailed comparisons of the kinetic states in Scheme II with the durations and conductance levels of closings selected on the basis of the durations of previous openings. For example, closings such as O_3-C_6 should not be associated with transitions through subconductance levels, if the subconductance levels are all associated with C_5. It is also possible that there might be slightly different conductance levels associated with some of the other kinetic states. In any case, the complexity of the subconductance levels in various channels (Auerbach and Sachs, 1983, 1984; Fox, 1987; Patlak, 1988; Bosma, 1989; Rae et al., 1990; Tyerman et al., 1992) suggests that the relationship between the various conductance levels and the kinetic states will be more complex than considered above.

This work was supported by grants from the National Institutes of Health (AR-32805) and the Muscular Dystrophy Association (to K. L. Magleby) and fellowships from a National Institutes of Health Training Grant (NS 07044) (to W. B. Ferguson and O. B. McManus).

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