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Preventing Errors When Estimating Single Channel Properties from the Analysis of Current Fluctuations

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ABSTRACT The conductance, number, and mean open time of ion channels can be estimated from fluctuations in membrane current. To examine potential errors associated with fluctuation analysis, we simulated ensemble currents and estimated single channel properties. The number (N) and amplitude (i) of the underlying single channels were estimated using nonstationary fluctuation analysis, while mean open time was estimated using covariance and spectral analysis. Both excessive filtering and the analysis of segments of current that were too brief led to underestimates of i and overestimates of N. Setting the low-pass cut-off frequency of the filter to greater than five times the inverse of the effective mean channel open time (burst duration) and analyzing segments of current that were at least 80 times the effective mean channel open time reduced the errors to <2%. With excessive filtering, Butterworth filtering gave up to 10% less error in estimating i and N than Bessel filtering. Estimates of mean open time obtained from the time constant of decay of the covariance, τ_{obs}, at low open probabilities (P_o) were much less sensitive to filtering than estimates of i and N. Extrapolating plots of τ_{obs} versus mean current to the ordinate provided a method to estimate mean open time from data obtained at higher P_o, where τ_{obs} no longer represents mean open time. Bessel filtering gave the least error when estimating τ_{obs} from the decay of the covariance function, and Butterworth filtering gave the least error when estimating τ_{obs} from spectral density functions.

INTRODUCTION

Fluctuations in membrane current arise in part from the opening and closing of ion channels. An important step toward understanding the properties of membrane currents is to determine the numbers, conductances, and lifetimes of the ion channels contributing to the currents (Hille, 1992). Prior to the advent of direct recordings from single channels with the patch clamp technique (Sakmann and Neher, 1983), analysis of the fluctuations in membrane current was one of the major means of estimating the properties of ion channels (reviewed by Neher and Stevens (1977) and DeFelice (1981)). In spite of the power of the single channel recording technique, fluctuation analysis is still widely used, since it is not always practical to record data from single channels, and whole cell recordings have revealed currents that arise from very small channels that can (at least for now) only be studied with fluctuation analysis (Cull-Candy et al., 1988; Bennett et al., 1989). Furthermore, fluctuation analysis can provide a rapid assessment of the actions of various channel modulators without the time consuming process of single channel recording and analysis. While direct comparisons of data obtained from fluctuation analysis and single channel recording have established the ability of fluctuation analysis to correctly reveal channel properties (Brum et al., 1984; Lux and Brown, 1984; Kimitsuki et al., 1990; Robinson et al., 1991), it is also known that there can be substantial errors (Gardner et al., 1984).

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A number of studies have discussed errors associated with fluctuation analysis and presented methods to test for and prevent them (Anderson and Stevens, 1973; Neher and Sakmann, 1976; Colquhoun and Hawkes, 1977; Neher and Stevens, 1977; Sigworth, 1980, 1981, 1985; Conti et al. 1980, 1984; DeFelice, 1981; Ruff, 1982; Lux and Brown, 1984; Holton and Hudspeth, 1986). One of the major contributors to error is the filtering applied to remove unwanted noise. Additional sources of error are the effects of open probability, P_o, on the estimates of mean channel open time and the analysis of segments of data of insufficient duration.

The purpose of this paper is to extend these previous studies by presenting some additional guidelines and methods for reducing errors when applying nonstationary fluctuation analysis, covariance analysis, and spectral analysis to continuous data. Errors associated with filtering, insufficient data length, and high P_o are characterized by studying ensemble currents generated from simulated single channel currents. Based on the findings, suggestions are made for selecting filters, minimizing filter-induced errors, determining the minimal durations of data to analyze, and estimating mean channel open time at high P_o. A preliminary report of some of these results has appeared (Silberberg and Magleby, 1993).

METHODS

Most computer programs for the generation and analysis of simulated whole-cell currents were written independently by each investigator using different algorithms. Results from the two independent analyses were then compared as an additional control on the calculations. Programs were either written in NDP FORTRAN and run on a Number-Smasher 860 board (Microway, Kingston, MA), or written in Quick Basic 4.5 (Microsoft Corp., Redmond, WA) and run on 486-based computers.

Generation of whole-cell currents and filtering

The simulation of single channel currents has been described previously (Neher and Stevens, 1977; Clay and DeFelice, 1983; Blatz and Magleby, 1986; Magleby and Weiss, 1990). Simulated whole cell (ensemble) currents were generated by summing from 1000 to 100,000 simulated single channel currents. Two different methods were used to generate filtered currents: the summation of filtered step responses (Colquhoun and Sigworth, 1983; Magleby and Weiss, 1990) which was equivalent to a four pole low-pass Bessel filter, and digital low-pass filtering of the summed current response.

For the digital filtering of summed current responses, the digital Gaussian filter described by Colquhoun and Sigworth (1983), which is equivalent to an infinite-pole low-pass Bessel filter, was used initially. To better mimic true experimental conditions, digital approximations of four-pole (−24 dB/octave) low-pass Bessel and Butterworth filters were implemented using published equations and coefficients (Tietze and Schenk, 1978). The level of filtering is expressed in this study as the cut-off (corner) frequency (f_c; in kHz) which gives a −3-dB response for a sine wave. The cut-off frequency is defined as the frequency of a sine wave at which the filtered sine wave amplitude is attenuated to 0.707 of the unfiltered sine wave amplitude (−3 dB = 20 log (0.707/1.0)).

The Bessel and Butterworth digital filters were found to be well calibrated. For a given cut-off frequency setting of the filters, a sine wave of the same frequency was attenuated to within 0.2% of the 0.707 value expected for −3-dB attenuation. This is shown in Fig. 1 *A*, which plots the amplitude response of a sine wave against the normalized cut-off frequency for both digital filters. The dashed line indicates the −3-dB point. Frequencies just below the cut-off frequency are attenuated more for the Bessel than for the Butterworth filter, and frequencies above the cut-off frequency are attenuated less for the Bessel than for the Butterworth filter. For example, if the cut-off frequencies of both a Bessel and Butterworth filter are set to 1 kHz, then a 2-kHz sine wave will be attenuated to only 0.21 of its original amplitude for the Bessel filter compared to 0.062 for the Butterworth filter.

Bessel filters are generally preferred over Butterworth filters for the analysis of single channel current records, since they are better at preserving the shape of the single channel response (Colquhoun and Sigworth, 1983). This is shown in Fig. 1 *B*, which plots the responses with digital Bessel (*thick lines*) and Butterworth (*thin lines*) filtering of a rectangular pulse for the indicated levels of filtering. (A rectangular pulse would be similar to an idealized channel opening in a single channel record.) For the same −3-dB cut-off frequency setting for a sine wave, the response for Bessel filtering rises more quickly than for Butterworth filtering and has minimal overshoot (ringing) compared to the pronounced overshoot on the Butterworth filter. The Butterworth filtered response

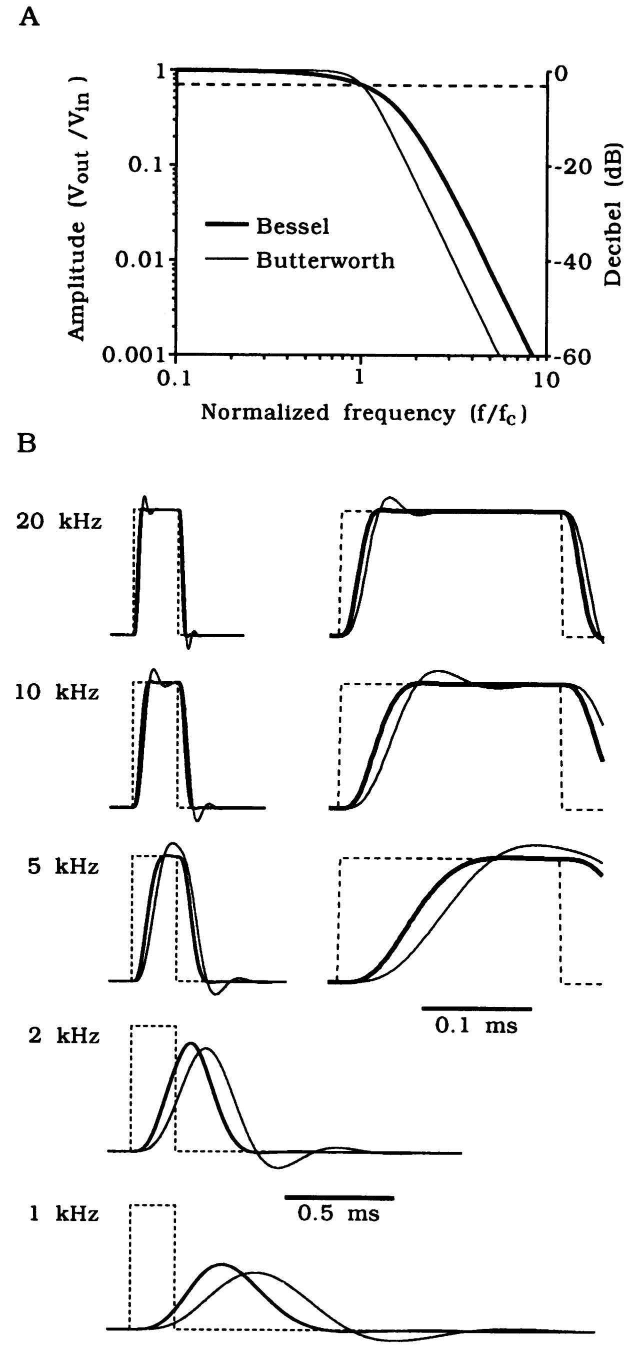

FIGURE 1 Comparison of the characteristics of digital Bessel and Butterworth four-pole low-pass filters. (*A*) Plot of the fractional reduction in the amplitude of a filtered sine wave against the ratio of the sine wave frequency to the −3-dB cut-off frequency of the filters. The frequency response curves were determined by examining the response at over 200 different frequencies for each filter. (*B*) Filtered responses for a unitary pulse of 0.2-ms duration for the indicated cut-off frequencies. Bessel, thick lines; Butterworth, thin lines.

rises less steeply than the Bessel filtered response because the Butterworth gives a greater attenuation of the high frequencies (Fig. 1 *A*).

In comparison to analog filters, the output for digital Bessel and Butterworth filters has an exaggerated overshoot to an input square wave if the sampling rate is less than about 20 times the cut-off frequency. This is the case because simulated single channel current events without filtering rise infinitely fast, violating a basic precondition for accurate digital filtering, which requires that the input pulse be band limited (Tietze and Schenk, 1978). To avoid this problem the sampling rate for the digital filtering was at least 20 times the cut-off frequency, unless specifically indicated. Such high sampling rates would not necessarily be needed for the analysis of experimental data with digital filters because of the prefiltering by the patch clamp amplifiers and recording systems.

Dead time and level of filtering

The level of filtering is expressed as the cut-off frequency (f_c) which gives a −3-dB response for a sine wave (see above) or as the dead time. For a Gaussian filter, which is equivalent to an infinite pole Bessel filter, the dead time (T_D; in milliseconds) is given by Colquhoun and Sigworth (1983),

$$T_D = A/f_c, \tag{1}$$

where $A = 0.179$ for a Gaussian filter and 0.187 for the four-pole digital filter used in this study. Dead time provides a useful definition of the level of filtering when dealing with step responses, such as single channel currents, since it indicates the actual duration of a channel opening that would give a half-amplitude response with filtering. Note that sampling at >3.6 samples per dead time meets the requirement of sampling at >20 times the cut-off frequency.

When comparing results in this study with experimental results obtained using commercial low-pass filters, it is important to note that the results in this study are based on four-pole (24 dB/octave) low-pass filters. It is also important to note that the frequency setting on the face of commercial filters may not be the −3-dB cut-off frequency shown in Fig. 1*A* by the dashed line. For example, for the Ithaco 4302 filter (Ithaco Inc, Ithaca, NY) the frequency setting on the dial gives the −3-dB frequency in the Butterworth (normal) mode and the −8.4-dB frequency in the Bessel (pulse) mode. The setting on the face of the filter can be multiplied by 0.623 to obtain the −3-dB cut-off frequency for the Bessel mode. The actual −3-dB cut-off frequency of low-pass commercial filters can be checked for each dial setting by finding the frequency of a sine wave that is attenuated to 0.707 (−3 dB) of its unfiltered amplitude.

Unless otherwise indicated, four-pole (24 dB/octave) digital Bessel filtering was used in the simulations.

Noise

For some simulations, noise was added to the simulated ensemble currents to investigate the effects of noise on the results. The noise used was obtained from experimental whole-cell current records in the apparent resting state and in the absence of agonist and in some cases from patch clamp currents from excised patches of membrane in the absence of obvious channel activity. A wide range of noise magnitudes were examined, with the maximum variance of the noise equal to the variance of simulated ensemble currents with an open probability of 0.4.

Estimation of *i* and *N*

If variations in membrane current are assumed to arise from a homogeneous population of *N* independent channels with a single conducting level, the variance of the current (σ^2) is related to mean current (I) by

$$\sigma^2 = iI - I^2/N, \tag{2}$$

where the number of channels *N* and the single channel current *i* can be estimated by fitting the relationship between σ^2 and I over a range of mean currents (Sigworth, 1980). Estimating *N* and *i* in this manner (nonstationary fluctuation analysis) was first used to estimate the properties of sodium channels at the node of Ranvier (Sigworth, 1980). The nerve was repeatedly activated by voltage steps and the current measured at fixed times after the onset of the step. The fluctuation of the current at fixed times after the onset of the step was then used to estimate the variance of the current as a function of the mean current. It was assumed that the open probability of the channels at a given time after the onset of the step was the same from trial to trial and that the changes in the mean current reflected changes in the open probability.

In recent years, nonstationary fluctuation analysis has been used to study agonist activated channels by calculating the mean and variance of short segments of continuously recorded currents activated by different concentrations of agonist (McMahon et al., 1989; Huettner, 1990; Owen et al., 1990; Grissmer et al., 1992). It is this application of nonstationary fluctuation analysis that we examine in this study by analyzing segments of simulated currents generated from summed single channel current records. Summed (ensemble) currents were generated with different *Po* by varying one of the rate constants for the generation of the single channel currents. The variance for each of the ensemble currents was then plotted against the mean of that ensemble current, and the results for the various ensemble currents were fit to Eq. 2 using the Marquardt-Levenberg algorithm for least squares (SigmaPlot 5.0; Jandel Scientific, San Rafael, CA). Simulated ensemble currents ranged from 25 ms to 8 s in duration.

Estimation of mean channel open time at low P_o

Mean channel open time was estimated from simulated currents at low P_o either from the time constant of decay of semilogarithmic plots of the covariance function or from the half-power point of Lorentzian fits to power spectra (Anderson and Stevens, 1973; Neher and Sakmann, 1976; Colquhoun and Hawkes, 1977; Neher and Stevens, 1977; Magleby and Weinstock, 1980; DeFelice, 1981) The spectral and covariance functions contain the same information in different forms. Covariance $C_{t'}$ was calculated from

$$C_{t'} = \lim_{T\to\infty} \frac{1}{T} \int_0^T \Delta I(t)\Delta I(t+t')\, dt \qquad (3)$$

where T is the integration time, $\Delta I(t)$ and $\Delta I(t+t')$ are the deviations from the mean current at time t and $t+t'$, respectively. The covariance function describes the correlation between the deviation from the mean current at a specific time t, and at a later time $t+t'$. Intuitively, if a channel is open at time t, the probability that the channel is still open at time $t+t'$ will depend on the mean channel open time. At low P_o, if the channel open times are exponentially distributed, $C_{t'}$ will decay exponentially as a function of the interval t' with a time constant equal to the mean channel open time.

For some simulations the covariance used to estimate the mean open time was the average of 100 determinations from 125-ms records of ensemble currents. In others the covariance was calculated from a single ensemble current of typically 2–8 s in duration. Similar results were obtained with both methods. The fits to the decay of the covariance were started at an interval equal to two dead times to exclude the distortion introduced by the filtering at shorter times. The fit was then typically extended to intervals equal to about two to three times the mean channel open time. With heavy filtering, the covariance function no longer decayed exponentially, as will be apparent in the figures in later parts of the paper. In these cases the fit was still started at an interval equivalent to two dead times and typically extended for about one mean open time.

Spectral density functions were obtained from the ensemble current traces using the fast Fourier transform, as described in Press et al. (1986). Mean channel open times were then estimated from the spectral density obtained at low P_o by fitting the curves with a Lorentzian:

$$S(f) = S_1(0)/[1 + (f/f_c)^2], \qquad (4)$$

where f is frequency, $S_1(0)$ is the spectral density for $f = 0$, and f_c is the frequency at which $S(f_c)$ is half of the value of $S_1(0)$. The time constant τ_{obs}, which gives an approximation of the mean channel open time at low P_o, was then determined from the corner frequency as:

$$\tau_{obs} = 1/2\pi f_c. \qquad (5)$$

Repeatability of observations

When the analyzed current records were of sufficient duration (see Results), stochastic variation in the estimates of i, N, and mean channel open time were negligible, as indicated by repeatable findings with different random number seeds. For ease of comparison between filtered and unfiltered data, the results with filtering are typically expressed as a percentage of the results without filtering. When investigating the effects of current duration where stochastic variation could have effects because of the limited durations of the analyzed currents, the entire analysis was repeated numerous times and error bars included to indicate the uncertainty in the estimates.

RESULTS

Filtering reduces the magnitude of current fluctuations

Fig. 2*A*, upper four traces, presents simulated single channel currents for the two state model described by

$$C \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} O$$

SCHEME I

where O and C are the open and closed states, and α and β are 5000/s and 1250/s, respectively, giving mean open and closed lifetimes of 0.2 and 0.8 ms. The unitary (single channel) current is 1 pA. The bottom trace in Fig. 2*A* shows the ensemble of 1000 such traces. Fluctuations of the current

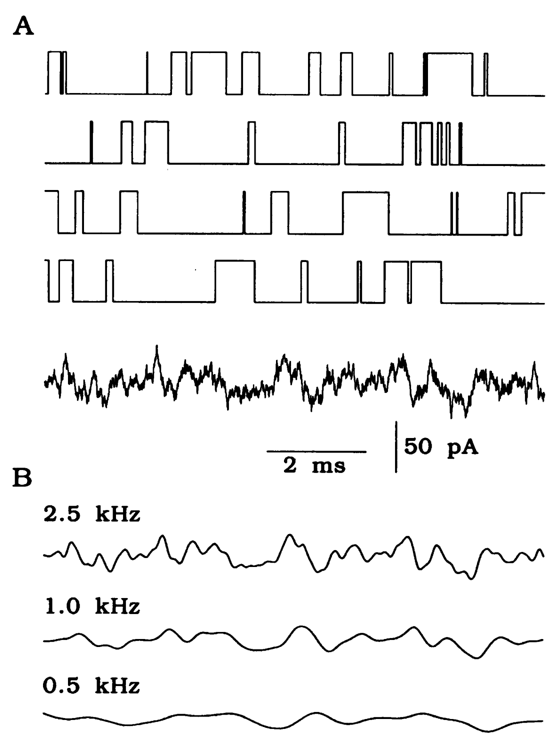

FIGURE 2 (*A*) Simulation of ensemble currents. The upper four records present unfiltered single channel current traces for a two-state model (Scheme I) with a unitary current, *i*, of 1 pA, a mean open time of 0.2 ms, and a P_o of 0.2. Channels open upward. The bottom record presents the sum (ensemble current) of 1000 single channel current traces. (*B*) The effect of 2.5-, 1.0-, and 0.5-kHz filtering of the ensemble current shown in *A*. Lowpass digital Bessel filter. All traces were sampled at an effective frequency of 500 kHz.

resulting from the stochastic properties of the individual channels are clearly evident.

It is well known that filtering reduces the magnitude of current fluctuations. Fig. 2 *B* demonstrates this effect on the simulated ensemble current shown in Fig. 2 *A* using a low-pass digital Bessel filter. The reduced amplitudes of the current fluctuations with filtering would be expected to introduce errors into estimates of single channel parameters obtained from fluctuation analysis.

Filtering reduces the variance of current fluctuations

Fig. 3 plots the relationship between the variance and the mean current for ensemble currents generated by summing 2000 single channel current traces simulated with Scheme I. The channels had a mean open time of 0.2 ms and a unitary current of 1.0 pA. To obtain a range of mean currents, each ensemble current was generated using a different opening rate β. Since the mean current is proportional to the open probability (P_o), both mean current and P_o are plotted. The observed parabolic relationship between variance and mean current in the absence of filtering (*filled circles*) can be intuitively understood by examining the extreme conditions.

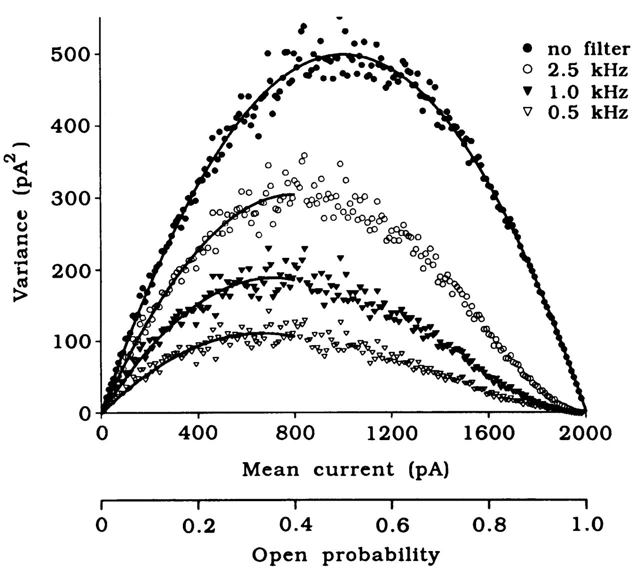

FIGURE 3 Effect of filtering on plots of variance versus mean current for ensemble current traces generated for a two-state model. The unitary current was 1 pA and mean open time was 0.2 ms. Each filled circle denotes the relationship between the variance and the mean current of unfiltered ensemble current traces generated by summing 2000 single-channel current traces 125 ms in duration. Ensemble currents were generated and analyzed for 198 P_o ranging from 0.005 to 0.99 at 0.005 increments. The continuous line through the filed circles is the fit with Eq. 2, giving estimated values of *i* and *N* of 0.996 pA and 2010 channels, respectively. The ensemble currents were then filtered at different levels with a digital four-pole Bessel filter, and the variance and mean of the current traces were recalculated and plotted. The continuous lines through the filtered estimates are the fit with Eq. 2 up to a P_o of 0.4, giving estimated values of *i* of 0.76, 0.53, and 0.34 pA and estimated values of *N* of 2100, 2660, and 3935 channels for 2.5-, 1.0-, and 0.5-kHz filtering, respectively. Data were sampled at 100 kHz.

Fluctuations in the ensemble current trace are not expected when all channels are closed ($P_o = 0$) or when all channels are continuously open ($P_o = 1$). Hence, the variance is zero under these conditions. Maximal variance is predicted for a P_o of 0.5, since the channels spend equal amounts of time at the two extreme current levels (open and closed), maximizing the fluctuations.

The simulated current fluctuations without filtering are well fit by Eq. 2 (*continuous line* through *filled circles*), giving an estimated unitary current *i* of 0.996 pA and an estimated number of channels *N* of 2010. These values are almost identical to those used to simulate the currents (1.0 pA and 2000 channels). Thus, nonstationary fluctuation analysis can obtain accurate estimates of *i* and *N* in the absence of filtering. With filtering, however, the parabolic relationship between the variance and mean current became attenuated and skewed toward lower mean current values. Consequently, maximal variance occurs at a P_o less than 0.5.

Filtered data can underestimate unitary conductance and overestimate the number of channels

Fitting the filtered curves in Fig. 3 up to a P_o of 0.4 with Eq. 2 gave estimates of *i* of 0.76, 0.53, and 0.34 pA and estimates of *N* of 2100, 2660, and 3935 channels, for 2.5-, 1.0-, and 0.5-kHz filtering, respectively (*continuous lines*). Thus, increased levels of filtering led to progressive underestimates of *i* and overestimates of *N*. Fitting beyond a P_o of 0.6 produced even greater errors in the estimates due to the skew of the variance-current plot. For example, fitting the data filtered at 2.5 kHz up to a P_o of 0.9 gave estimated values of *i* and *N* of 0.69 pA and 2600 channels, compared to the values of 0.76 pA and 2100 channels when fitting up to a P_o of 0.4. Hence, to reduce the effects of the skew on the estimates of *i* and *N*, the fitting of filtered data in the remainder of the paper will be up to P_o values of 0.4.

Fig. 4 plots estimates of *i* and *N* for a range of mean open times and filtering. For each plotted point, estimates of *i* and *N* were made as shown in Fig. 3 using nonstationary fluctuation analysis of currents generated at a series of open probabilities. For a channel with a mean open time of 0.1 ms, 1-kHz filtering led to a 66% underestimation of *i* and a 90% overestimation of *N*. Increasing the cut-off frequency to 5 kHz reduced the errors to 25 and 9%, respectively. Increasing the mean open time reduced the errors at all levels of filtering.

Fig. 4 shows that the cut-off frequency of the low-pass filter should be greater than five times the inverse of the mean open time to keep errors in estimates of *i* to less than 2–3%. For example, for a mean open time of 1 ms the cut-off frequency should be 5 kHz or higher, or equivalently, the mean open time should be greater than about 25 dead times. Estimates of *N* are less sensitive to filtering. Thus, if the errors in *i* are less than 2–3%, the errors in *N* should be negligible.

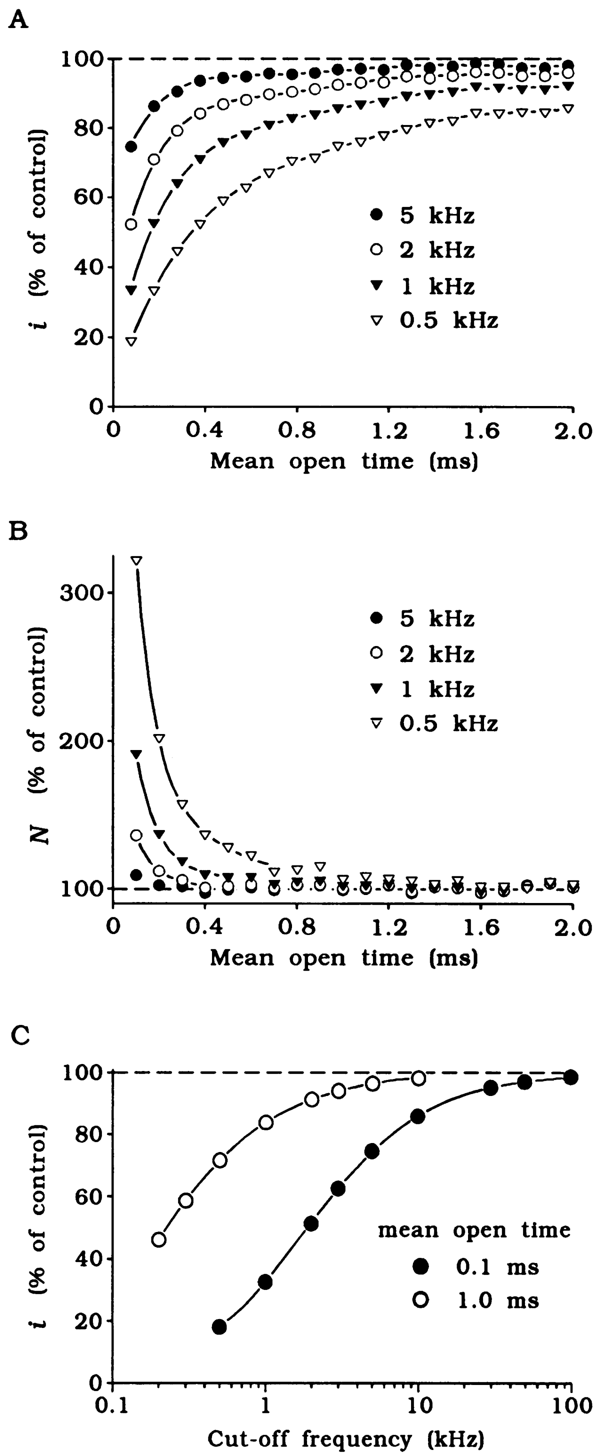

FIGURE 4 Effect of filtering on the estimates of unitary current i, and number of channels N, obtained with nonstationary fluctuation analysis. Ensemble currents were generated using the same parameters as in Fig. 3. Filtering was with a digital Bessel filter. Estimates of i (A) and N (B) as a function of mean channel open time, expressed as percent of the estimated values without filtering. Each plotted estimate of i and N was determined from fitting the relationship between the variance and the mean current using Eq. 2, as demonstrated in Fig. 3. (C) Estimates of i as a function of filter cut-off frequency for simulated currents generated for single channels with a mean open time of 0.1 ms (*filled circles*) or 1.0 ms (*open circles*). The continuous lines through the data points were drawn by eye for all parts of the figure.

Since estimates of i obtained by nonstationary fluctuation analysis are especially sensitive to filtering, errors in estimates of i were examined in greater detail. Results are shown in Fig. 4 C, which plots estimates of i against the cut-off frequency, for a two state channel with different mean open times. For a mean open time of 0.1 ms, i was underestimated by greater than 50% with 2-kHz filtering, and was still underestimated by 3% with 50-kHz filtering (*filled circles*). For a mean open time of 1.0 ms the errors were less, but still considerable for cut-off frequencies of less than 3 kHz (*open circles*). For any mean open time, the cut-off frequency giving the same errors in estimating i as indicated by the open circles in Fig. 4 C can be determined by multiplying the values on the abscissa by: 1.0 ms/(desired mean open time in milliseconds).

Factors contributing to the underestimation of *i*

To test whether the underestimation of i resulted from attenuation of peak single channel current induced by filtering, we simulated ensemble currents using fixed channel open times so that all channel openings would be attenuated an identical amount with filtering. The filled circles in Fig. 5 A plot the results of simulations with a fixed open time of 0.2 ms and no filtering. The unfiltered data were well described with Eq. 2 (*continuous line*), giving estimated values of i and N of 1.001 pA and 1996 channels, almost identical to the values of 1.0 pA and 2000 channels used for the simulations.

With 2-kHz filtering (dead time of 0.09 ms), the single channel currents for the 0.2-ms openings were attenuated by 13.2% (*inset*, Fig. 5 A). However, the value of i estimated from ensemble currents with the same level of filtering was underestimated by 37.5% (determined by fitting Eq. 2 to the open circles in Fig. 5 A). Thus, the under estimation of i is greater than the attenuation of the peak single channel current. This raises the possibility that current broadening induced by the filter also contributes to the errors. To test this idea, ensemble simulated currents were filtered at 3.5 kHz so that the peak of the filtered single channel current was the same amplitude as the unfiltered single channel current (Fig. 5 B, *inset*). Even though the peak was not attenuated, filtering at 3.5 kHz resulted in an underestimation of i by 21% (determined by fitting Eq. 2 to the open circles in Fig. 5 B). Thus, filtering reduced estimates of i due to both broadening and attenuation of the single channel currents.

Effect of filtering on estimates of *i* and *N* for a three-state model

It is clear from Fig. 4 that, at least for a two-state model, the errors in estimating i and N with Eq. 2 depend strongly on the relationship between the duration of channel opening and the extent of filtering. To examine whether the effect of filtering differs for more complex kinetic schemes, we analyzed

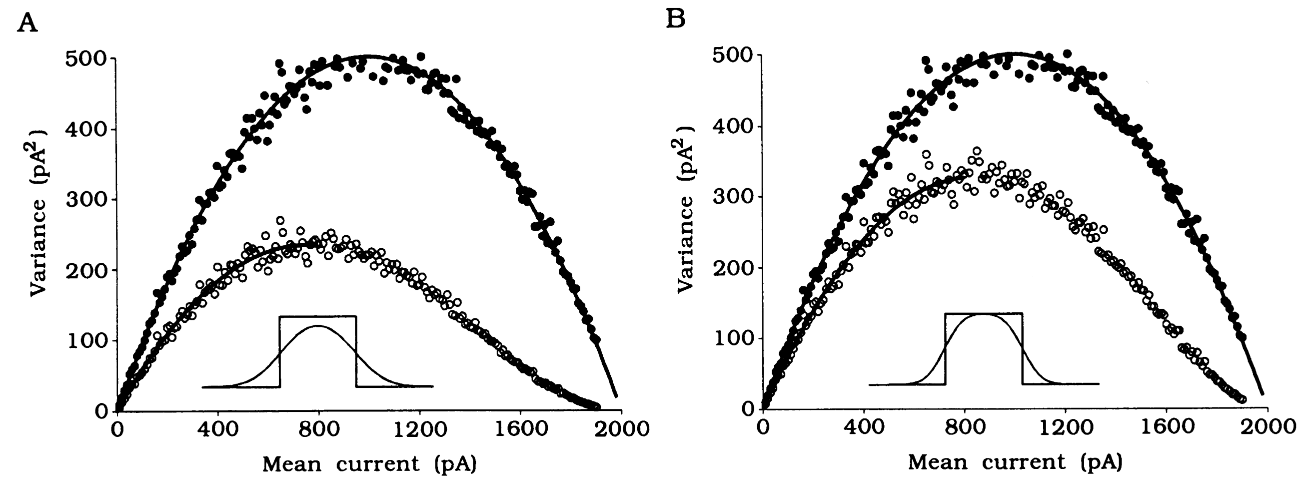

FIGURE 5 Filtering decreases estimates of i by reducing the peak unitary current and broadening the response. (*A*) The estimated value of i is less than predicted from the attenuation of peak unitary current by filtering. Ensemble currents were simulated with single channel openings with a fixed duration of 0.2 ms so that all isolated openings would be attenuated the same amount with filtering. The single channel current amplitude was 1 pA, and the channel closed times were exponentially distributed. The filled circles plot the relationship between the variance and mean current of unfiltered ensemble currents, and the open circles plot the results for filtering at 2 kHz. The peak unitary current was reduced to 0.87 pA with 2-kHz filtering (*insert*, the filtered current trace was time-shifted to overlap the unfiltered current to better illustrate the relationship between the two current traces). The continuous lines are fits with Eq. 2, giving estimated values of i of 1.001 and 0.625 pA for the unfiltered and filtered current traces, respectively. Ensemble currents were generated from 2000 single-channel current traces, 125 ms in duration. (*B*) Filtering that did not reduce the peak amplitude of the filtered single channel current still reduced the estimate of i. Same parameters and seed number for the random number generator as for *A*, except that 3.5-kHz filtering was selected so that the peak of the filtered current was not reduced; i estimated by fitting with Eq. 2 was 0.79 pA (*continuous line* through the *open circles*). Data in *A* and *B* were sampled at 100 kHz and filtered with a digital Gaussian filter.

ensemble currents simulated for the three-state model described by Scheme II,

$$C \underset{k_2}{\overset{k_1}{\rightleftharpoons}} C \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} O$$

SCHEME II

with β fixed at 10,000/s and k_2 fixed at 3000/s to give bursts of closely spaced channel openings. On average, a burst consisted of 4.3 channel openings interrupted by 3.3 brief closures (flickers) with a mean duration of 0.077 ms. Mean channel open time was altered by varying α, and for a given α, P_o was altered by varying k_1.

Fig. 6 *A* illustrates a relatively long burst of channel activity before (*upper trace*) and following (*lower trace*) 2-kHz filtering with a digital Bessel filter. Fig. 6, *B* and *C*, shows the effect of filtering on estimates of i and N for the three-state model. Estimates of i and N were made using nonstationary fluctuation analysis, as shown in Fig. 3. For the same mean open times, filtering introduced less error in the estimates of i and N for the bursting model described by Scheme II than for the two-state model described by Scheme 1 (compare Fig. 6 to Fig. 4). The reason for this is that, after filtering, brief closed events often go undetected resulting in longer effective mean open times for the bursting model (Fig. 6 *A*, *lower trace*).

A comparison of the effects of Bessel and Butterworth filters on estimates of *i* and *N*

Fig. 7 compares the effects of Bessel and Butterworth filtering on estimates of i and N obtained for a two-state model with nonstationary fluctuation analysis. In general, if the filtering was excessive compared to the mean open time of the channel, then the errors tended to be less with Butterworth filtering than with Bessel filtering. If the filtering is not excessive, then either filter should be satisfactory.

Errors associated with applying nonstationary fluctuation analysis to short segments of data

Nonstationary fluctuation analysis can be applied to agonist activated currents by determining the variance and mean of segments of steady-state current obtained at different P_o due to different concentrations of agonists. Alternatively, if the current activates slowly enough, short segments of current recorded during the activation can each be assumed to represent a different P_o. Using either approach, if the segments of analyzed data are too short, the variance for any given segment will, on average, be underestimated, because the mean current for that segment is a local mean which minimizes the variance of the given segment of data (Neher and Stevens, 1977; Conti et al., 1980; DeFelice, 1981). Even small errors in the mean can lead to large errors in the estimated variance when the fluctuations about the mean are

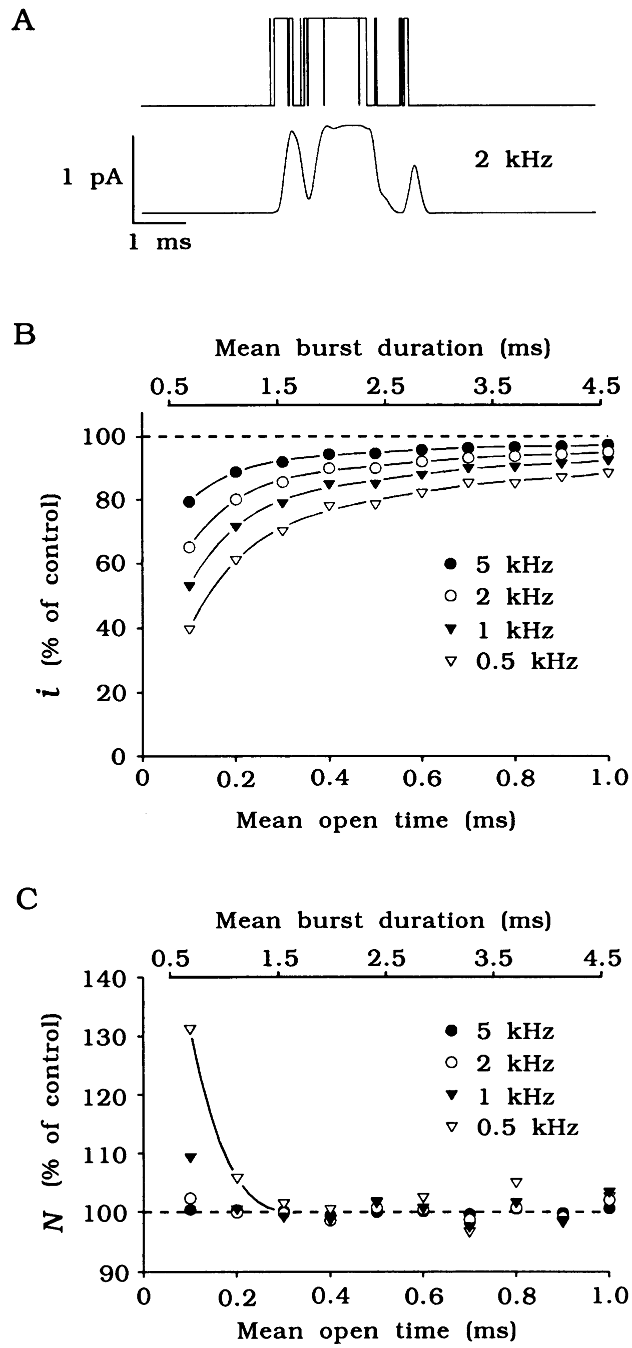

FIGURE 6 Effect of filtering on the estimates of i and N from ensemble currents generated for a three-state model (Scheme II). (*A*) Bursts of channel activity prior to (*top trace*) and following (*bottom trace*) 2-kHz filtering with a digital Bessel filter (mean channel open time of 0.2 ms). (*B* and *C*) Estimates of i and N as a function of mean channel open time (*lower abscissa*) and mean burst duration (*upper abscissa*), expressed as percent of the estimated values without filtering. N and i were estimated from fitting the relationship between the variance and the mean current using Eq. 2, as demonstrated in Fig. 3. The continuous lines were drawn by eye.

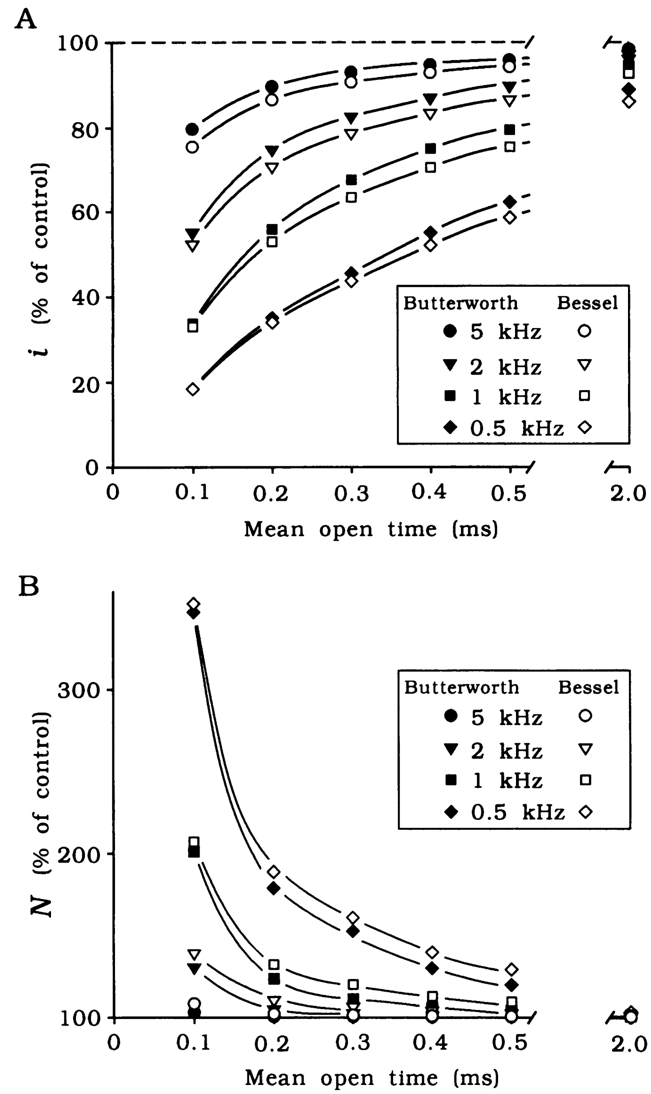

FIGURE 7 Comparison of estimates of i and N obtained with Butterworth (*filled symbols*) and Bessel (*open symbols*) four-pole digital filters. Estimates were determined with nonstationary fluctuation analysis, as shown in Fig. 3. Plotted values are expressed as a percentage of the estimated values without filtering.

small. For channels with long effective mean open times (long burst durations), the current would fluctuate very slowly so that many hundreds of milliseconds of data would be required to obtain a representative estimate of the mean and variance (see Fig. 7 in Jonas and Sakmann (1992)).

To determine the effect of data segment duration on estimates of i and N, ensemble currents of different durations were analyzed. Ensemble currents of 3-s duration were first generated with Scheme II using rate constants of 1000/s, 10,000/s, and 1000/s for α, β, and k_2, respectively. The different open probabilities required for nonstationary fluctuation analysis were achieved by changing k_1. On average, these rate constants gave a burst of 11 channel openings interrupted by 0.09-ms closures. Each opening was, on average, 1 ms in duration resulting in an average burst duration of 11.9 ms. For each P_o, the variance and mean were determined by either analyzing the entire current trace or by analyzing segments of current ranging from 25 ms to 2 s in duration.

Fig. 8 presents the relationship between the duration of the analyzed current segments and the error in estimating i and N. The error bars in the figure represent the standard error of repeated estimates of i and N with nonstationary fluctuation analysis (up to 20 estimates were determined for the shortest current segments). Analysis of segments of current which were of insufficient length resulted in underestimates of i and overestimates of N. To reduce the error to less than 10%, current traces longer than 200 ms (17-fold longer than mean burst duration) were required for the examined kinetic scheme with a burst duration of 11.9 ms. To further reduce the error to less than 2%, current segments in excess of 1 s, more than 80-fold longer than the burst duration, were required. These observations are consistent with the calculation of Conti et al. (1980) that the current segments would have to be 60-fold longer than the effective correlation time of the fluctuations to reduce the underestimation of the variance to <3%. DeFelice (1981) also presents methods for calculating errors in variance.

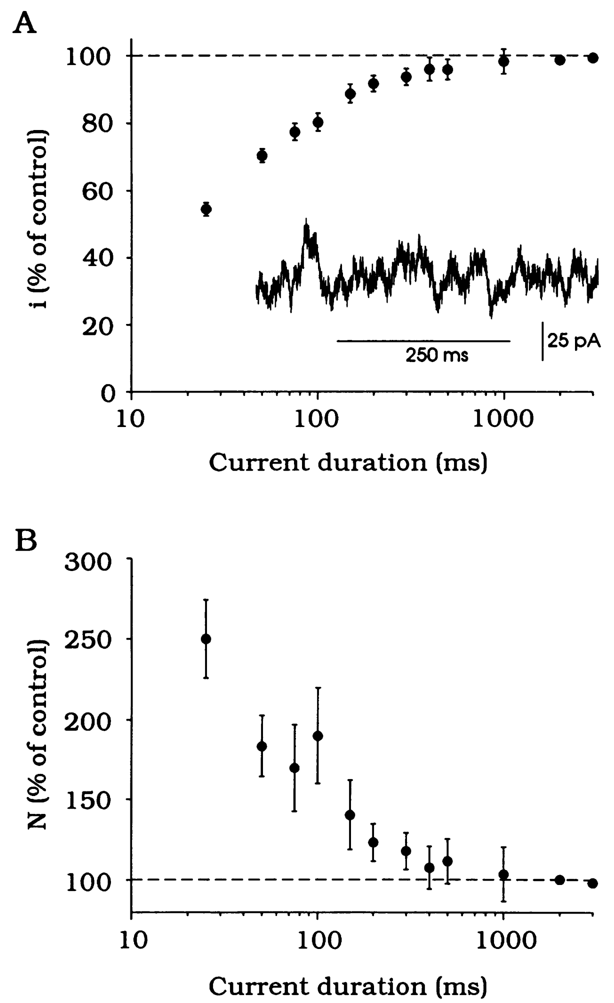

FIGURE 8 Effect of the duration of the current segments analyzed for nonstationary fluctuation analysis on estimates of i and N. Ensemble currents 3 s in duration were generated by summing 2000 single channel currents generated with Scheme II. Mean burst duration was 11.9 ms (see Results for rate constants). The inset in A shows a representative current trace obtained with a P_o of 0.05. A total of 100 ensemble currents were generated ranging in P_o from 0.01 to 0.4. The variance and mean at each P_o was either determined by analyzing the entire current trace or by analyzing segments of current ranging from 25 to 2000 ms in duration. The values of mean current and variance determined for a given current duration were used to estimate i and N with Eq. 2. For data segments shorter than 1500 ms, each filled circle is the mean (± SE) of multiple (3–20) determinations of i and N obtained by analyzing 100 current traces in each case. The results are normalized to the true values of i and N.

At first it might seem surprising that such long current segments are required to reduce the errors to low levels. However, examination of the fluctuating current record reveals that considerable durations of current would be required to obtain a representative estimate of the mean current and variance (*insert*, Fig. 8 *A*). The results in Fig. 8 place some rather severe experimental limitations, in the absence of corrections, on estimating properties of channels with prolonged effective mean open times. For example, if the effective mean open time (burst duration) of a channel were 250 ms (Schwartz, 1977), then (stable) segments of current at least 20 s in duration would have to be analyzed at each P_o to reduce the errors in estimates of i and N to <2%.

Errors in estimating mean channel open time from covariance analysis at high *Po*

Fluctuations from the mean current occur because of the stochastic variation in the number of channels open at any time. For the two-state model described by Scheme I the current then returns, on average, to the mean current level with a time constant τ, given by (Hodgkin and Huxley, 1952)

$$\tau = 1/(\alpha + \beta) \tag{6}$$

where τ can be extracted from the ensemble current using either covariance or spectral analysis. As the forward rate constant β becomes negligible in Scheme I and Eq. 6, P_o approaches zero and τ approaches the mean open time given by $1/\alpha$. Thus, at low P_o, $1/\alpha$ is approximated by τ.

To examine the magnitude of error in estimating mean open time when β is not negligible, estimates of τ were obtained from the covariance of the current fluctuations at different P_o (see Methods). Fig. 9 *A* presents semilogarithmic plots of the covariance of the ensemble current fluctuations for Scheme I with a mean open time of 0.1 ms and for P_o of 0.01 and 0.7. The observed time constants of the decays of the covariance (τ_{obs}) were 0.10 and 0.029 ms for the P_o of 0.01 and 0.7, respectively (*continuous lines*). Thus, τ_{obs} correctly estimated the mean open time when P_o was low (0.01) and underestimated the mean open time by 71% when the open probability was high (0.7).

Since τ_{obs} at both low and high P_o in Fig. 9 *A* are just as predicted by Eq. 6, it might be possible to estimate true mean open time from τ_{obs}.

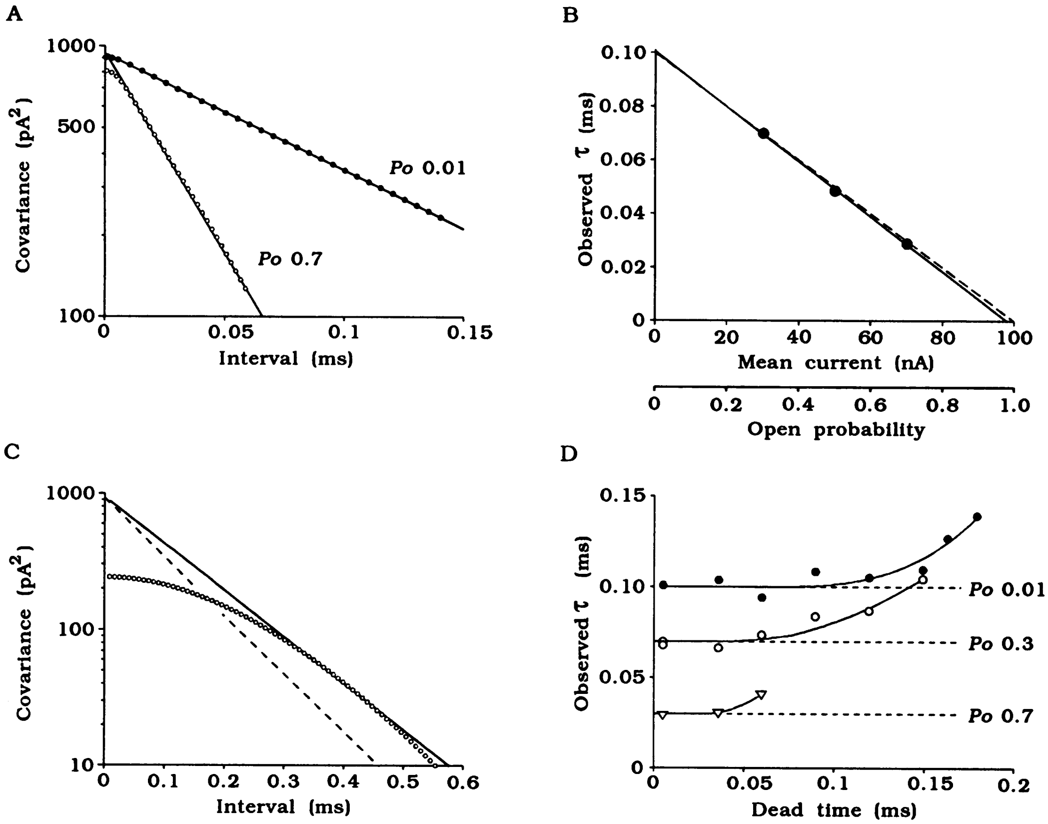

FIGURE 9 Effect of P_o and filtering on estimates of mean open time obtained with covariance analysis for ensemble currents generated with Scheme I with a mean open time of 0.1 ms and a unitary current of 1 pA. (*A*) Semilogarithmic plots of the covariance for P_o of 0.01 and 0.7. The time constants of decay of the covariance (*continuous lines*) were 0.101 for the P_o of 0.01 and 0.293 for the P_o of 0.7, in agreement with Eq. 6. The attenuated covariance at brief intervals arises from the 35.8-kHz filtering (dead time of 0.005 ms) used when generating the data. Hence, covariance values shorter than two dead times (0.01 ms) were excluded from the fitting. The covariance of the data obtained at the P_o of 0.7 (sum of 4000 single channel currents) were scaled for plotting by multiplying by 1.116 so that the intercept at the ordinate would match that of the data obtained at a P_o of 0.01 (sum of 100,000 single channel currents). (*B*) Plot of the time constant of decay of the covariance for three different P_o against the mean current. The continuous line, which is a regression fit to the data, gives an estimated mean open time of 0.1001 ms (intercept at ordinate). The dashed line is the theoretical relationship described by Eqs. 6 and 8. (*C*) Semilogarithmic plot of the covariance (*open circles*) and fit (*continuous line*), for a P_o of 0.01 and 1 kHz Bessel filtering (dead time of 0.18 ms). The dashed line plots the decay for 35.8 kHz filtering (from part *A*). The increased filtering increased the estimated mean open time from 0.1 to 0.14 ms. Ensemble currents of 100,000 channels in each case. For plotting, the data filtered at 1 kHz have been multiplied by 0.67 so that the intercept at the ordinate would match that of the dashed line. (*D*) Effect of Bessel filtering (expressed as dead time) and P_o on the time constants of decay of the covariance. The dashed lines plot the estimated time constants calculated with Eq. 6 for the indicated P_o and no filtering.

A method for estimating mean open time at high *Po*

For the two-state model described by Scheme I, the closed probability, $1 - P_o$, is given by

$$1 - Po = \alpha/(\alpha + \beta). \quad (7)$$

Combining Eqs. 6 and 7 and substituting τ_{obs} for τ gives

$$\tau_{obs} = (-1/\alpha)Po + 1/\alpha. \quad (8)$$

Hence, a plot of τ_{obs} vs. P_o should yield a straight line that intercepts the ordinate at the true mean open time of $1/\alpha$. The slope of the line will be $-1/\alpha$. Since P_o is proportional to the mean current, I, then

$$\tau_{obs} = (-1/\alpha)cI + 1/\alpha, \quad (9)$$

where c is a constant. Since it has been assumed that α is independent of P_o, then

$$\tau_{obs} = c'I + 1/\alpha, \quad (10)$$

where c' is a constant with a value of: $(-1/\alpha)c$. Thus, true mean open time can be estimated from the intercept at the ordinate of a plot of τ_{obs} versus mean current.

Fig. 9 *B* plots estimates of τ_{obs} versus mean current for three different P_o (*filled symbols*) for a two-state model with a mean open time of 0.1 ms and with 35.8-kHz filtering. (Also labeled on the abscissa is the open probability.) The continuous line, which is a regression fit to the plotted data, intercepts the ordinate at 0.1004 ms, giving an accurate estimate of the true mean open time. The dashed line plots the theoretical relationship described by Eq. 10 for the abscissa labeled mean current and by Eq. 8 for the abscissa labeled open probability. Thus, plots such as Fig. 9 *B* can provide a method for estimating true mean open time under conditions when data cannot be obtained at low open probabilities (see Discussion).

Filtering can lead to an overestimation of τ_{obs}

To examine the effect of filtering on the time constant of decay of the covariance, τ_{obs}, ensemble currents from simulated single channels with a mean open time of 0.1 ms were collected and analyzed with different levels of filtering. Fig. 9 *C* shows that filtering at 1.1 kHz (*open circles* fit by *continuous line*) increased τ_{obs} by 27% when compared to data filtered at 35.8 kHz (*dashed line*, obtained from the fit through the *filled circles* in part *A*). The extensive distortion at the beginning of the decay for the data filtered at 1.1 kHz resulted from the large dead time of 0.1627 ms associated with 1.1-kHz filtering. As in part *A*, the fitting was started at two dead times.

Fig. 9 *D* summarizes the effect of filtering on τ_{obs} for three different P_o. The dashed lines indicate the theoretical values calculated with Eq. 6. As the filtering and P_o were increased (increased dead times), τ_{obs} became overestimated.

Estimates of τ_{obs} were much less susceptible to error from filtering than estimates of i and N. For a P_o of 0.01, the error was less than about 10% as long as the mean open time was greater than the dead time. This can be compared to a 45% underestimation of i and a 30% overestimation of N for similar filtering. The data in Fig. 9 *D* can be scaled to different mean open times by multiplying the dead time by: ((desired mean open time in milliseconds)/0.1 ms). Thus, for a 1-ms mean open time and a P_o of 0.01, the dead time could be as large as 1 ms (cut-off frequency of about 200 Hz, Eq. 1) without appreciable error in estimates of τ_{obs}.

With filtering, some closings are missed (Fig. 6*A*), leading to an increased effective mean open time (Colquhoun and Sigworth, 1983; Blatz and Magleby, 1986). The maximum error contributed by missed events in Fig. 9 *D* for a P_o of 0.01 was calculated to be less than 2% at the higher levels of filtering, because so few of the closings were brief enough to be missed. Many more closings were missed for the data at a P_o of 0.3 and 0.7 at the higher levels of filtering because of the briefer closings. This may have contributed to the earlier deviation of τ_{obs} from the dashed lines when P_o was high.

Comparison of the effect of Bessel and Butterworth filters on estimates of mean channel open time

Fig. 10, *A* and *B*, shows the effect of Bessel (*open circles*) and Butterworth (*filled circles*) filtering on the covariance determined from ensemble currents simulated for Scheme I with a mean open time of 0.1 ms and an open probability of 0.01. Data for 5- and 2-kHz filtering are presented on linear plots, and the inserts present on semilogarithmic coordinates the portions of the same data that were fitted. With Bessel filtering the covariance decayed exponentially and the time constants of the decay of the covariance gave excellent estimates of the mean channel open time of 0.099 and 0.102 ms for the 5- and 2-kHz filtering, respectively. These estimates are within 2% of the 0.1-ms mean channel open time used to simulate the currents. In contrast, the ringing induced by the Butterworth filter distorted the covariance so that it no longer decayed exponentially. Consequently, there seemed little purpose in attempting to fit the covariance from the Butterworth filtered data. These findings suggest that Bessel filtering would usually be preferred for covariance analysis.

Channel life-times at low P_o can also be estimated from spectral density functions (Anderson and Stevens, 1973; Neher and Stevens, 1977). Spectral density functions are in the frequency domain, in contrast to covariance analysis, which is in the time domain. Butterworth filters are generally preferred over Bessel filters for analysis of power spectra since Butterworth filters are better at preserving the frequency information in the data at frequencies lower than the cut-off frequency. (Notice in Fig. 1 *A* that the Bessel filter starts to roll off at frequencies well below the corner frequency.) To test the effects of filtering on estimates of mean open time obtained with spectral analysis, ensemble current traces were generated as described for the covariance analysis, and the spectral density functions were calculated and fit by single Lorentzians (see Methods). For mean channel open times whose inverse is close to the cut-off frequency, Butterworth filtering gave less error than Bessel. For example, for a channel with a 0.1-ms mean lifetime and 5-kHz low pass filtering, spectral analysis of Butterworth filtered currents estimated the lifetime within 1% of the true lifetime, while spectral analysis of Bessel filtered currents overestimated the lifetime by 8%. (The fitting was extended to only 3 kHz to reduce the distortion from the roll-off of the filters.)

Thus, Bessel filtering is preferred with covariance analysis, and Butterworth filtering is preferred with spectral analysis. However, if the cut-off frequency of the filter is greater than about 10 times the frequency of the briefest inverse time constant in the data ($1/\tau_{obs}$), then filtering has little effect so that either type of filter can be used with either type of analysis.

Little effect of noise

Noise had little effect on estimates of i, N, and τ, as long as the variance (covariance for τ) of the noise in the absence of channel activity was subtracted from the variance (covariance for τ) of the ensemble currents of channel activity and noise. This would be expected since the variance of the noise and ensemble currents add separately. However, the lack of effect of noise for simulated data can be misleading, since noise could have large effects on experimental data if the properties of the noise changed as the conductance of the membrane changed with different levels of channel activity (Conti et al., 1984).

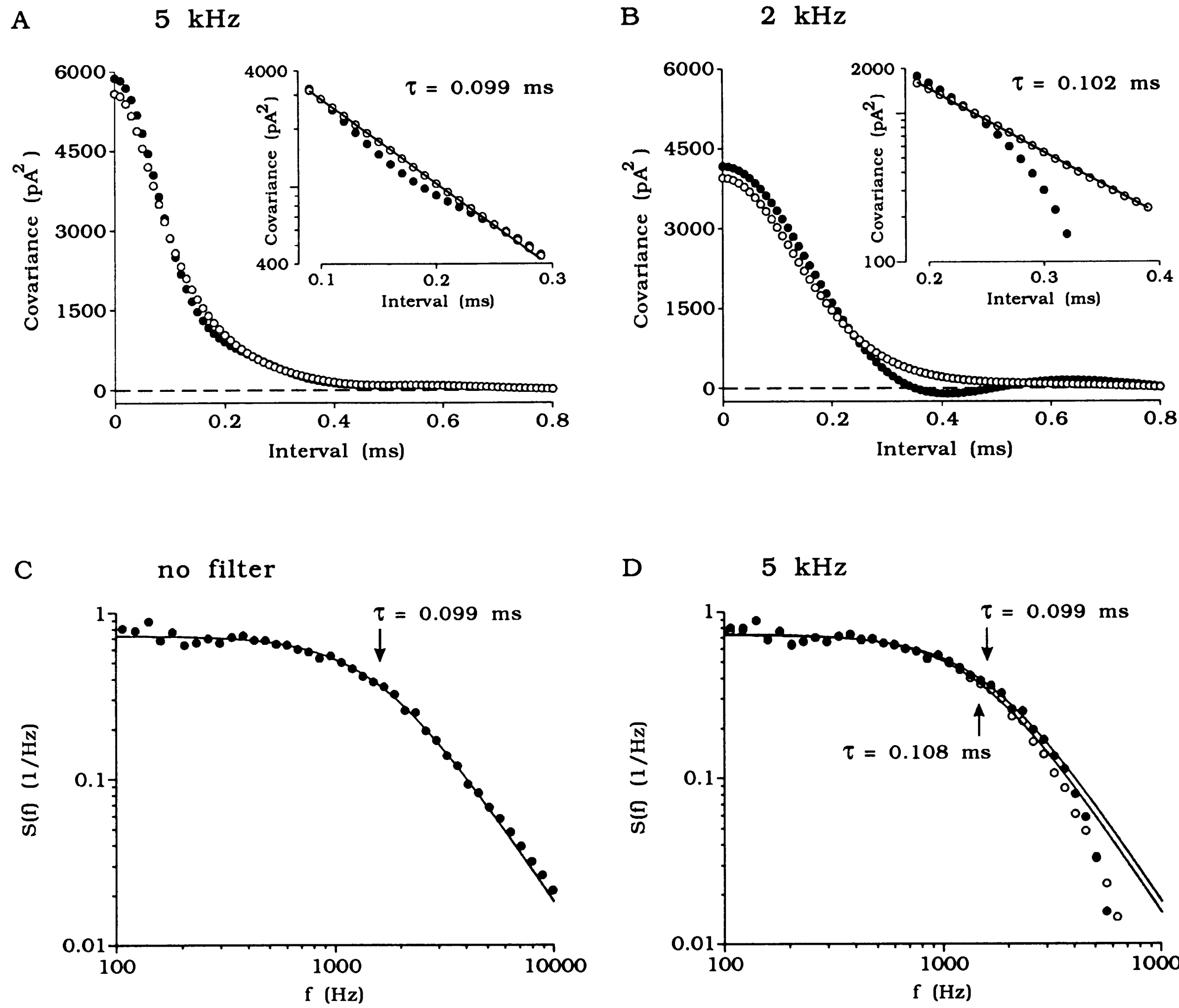

FIGURE 10 Butterworth filtering distorts the covariance of ensemble currents. Linear and semilogarithmic (*insert*) plots of the covariance for Bessel (*open symbols*) and Butterworth (*filled symbols*) filtering for ensemble currents generated using Scheme I with a mean open time of 0.1 ms and a P_o of 0.01. Plots are presented for 5 kHz (*A*) and 2 kHz (*B*) filtering. The Bessel filtered data were fit by a single exponential (*continuous line* in each *insert*), giving an estimated mean open time of 0.099 and 0.102 ms for the 5- and 2-kHz filtering, respectively. With the Butterworth filter, the covariance did not decay exponentially. Ensemble currents were generated from 30,000 single channel current traces, 125 ms in duration. Single channel current amplitude was 5 pA. (*C* and *D*) Power spectra of unfiltered (*C*) and filtered (*D*) ensemble currents generated for Scheme I with a mean open time of 0.1 ms and a P_o of 0.01. The data were fit by a single Lorentzian (*continuous lines*), giving an estimated mean open time of 0.099 ms for the unfiltered current (*C*) and 0.099 and 0.108 ms for 5-kHz filtering (*D*) with either Butterworth (*filled symbols*) or Bessel filters (*open symbols*), respectively. The data were fit up to 3 kHz. The power spectra were normalized by dividing by the variance (Press et al., 1986). The ensemble current was generated from 30,000 single channel current traces 82 s in duration. Sampling was at 20 kHz.

DISCUSSION

Filter-induced errors in estimating *i* and *N* with nonstationary fluctuation analysis

Filtering can lead to large underestimates of *i*, the unitary (single channel) current, determined with nonstationary fluctuation analysis (Figs. 3 and 4). The briefer the mean open time, the greater the error. For a two-state model, the cut-off frequency of the low-pass filter should be greater than five times the inverse of the mean open time to keep errors in estimates of *i* to less than 2–3%; i.e., 5 kHz for a 1-ms mean open time. In setting the final filter it is important to consider the contribution to the filtering of all the components in the system, including the patch clamp amplifier (voltage clamp) and the recording and storage devices. The effective cut-off frequency for a cascade of filters can be calculated from equations presented by Colquhoun and Sigworth (1983).

The underestimation of *i* was greater than the filter-induced attenuation of the peak single channel current (Fig. 5). Simulations with channel openings of fixed duration suggested that broadening of the unitary current steps by the filtering also contributed to the errors. The more slowly rising and decaying phases of the broadened current steps can be viewed as an envelope of an infinite number of subconductance levels. Multiple subconductance levels would lead to errors, since nonstationary fluctuation analysis assumes a single conducting state. In addition, the broadened unitary current steps can overlap, further reducing the variance. The greatly reduced variance at high P_o, resulting from this overlap, would contribute to the skewness in the relationship between the variance and mean current that is observed with filtering (Figs. 3 and 5).

In addition to the underestimates of i, excessive filtering also produces overestimates of N, the number of channels contributing to the ensemble current. In general, filter-induced errors in estimating N were less than errors in estimating i (Figs. 4 and 6). These differences in error may reflect the fact that, for a given P_o, the variance increases linearly with N and as the square of i. This can be seen by substituting iNP_o for I in Eq. 2, as shown by Sigworth (1980).

An observed skewness in a variance versus mean current plot for experimental data could suggest that the filtering is excessive in relationship to the mean open time. If such a skewness is observed, then the error in estimates of i and N can be partially reduced by fitting only the ascending limb of the variance versus mean current plot to avoid the most pronounced skewness. Excessive filtering would not give rise to a skewness under the rather unlikely situation where the sum of the opening (β) and closing (α) rate constants for each P_o is the same as the sum of the opening and closing rate constants at $1 - P_o$. For example, with this restriction the current fluctuations (and reduced variance introduced by filtering) would be identical at a P_o of 0.1 and 0.9.

Errors in estimating *i* and *N* induced by analyzing current records of insufficient duration

If the durations of the segments of currents analyzed for nonstationary fluctuation analysis are too brief compared to the effective mean lifetime (or burst duration) of the channel, then the variance for any given segment of data will, on average, be underestimated (Neher and Stevens, 1977; Conti et al., 1980; DeFelice, 1981). The mean current for that segment will be a local mean which minimizes the variance of the given segment of data. Underestimation of variance leads to an underestimation of i and overestimation of N (Fig. 8). For the examined three-state model, the durations of each segment of current had to be at least 80-fold longer than mean burst duration in order to reduce the errors to less than 2–3%.

In practice, it may be difficult to obtain stable agonist activated current records of sufficient duration for error free analysis because of activation and desensitization. Consequently, it may be necessary to fit the overall current record with a smoothing function to determine the best estimate of the mean current at each point in time. The variance would then be calculated about the mean estimated in this manner when analyzing segments of current.

Analysis of data with both excessive filtering and with current segments of insufficient duration could lead to pronounced underestimates of i and overestimates of N, since the errors are, to a first approximation, additive.

Filter-induced errors in estimating the time constant of decay of the covariance

The time constant of decay of covariance is determined by both the opening (β) and closing (α) rate constants for a two-state model (Scheme I and Eq. 6). Filtering can lead to an overestimation of the time constant of decay. The higher the P_o, the greater the error (Fig. 9). The greater error with higher P_o may result because the overlap of the filtered channel openings leads to decreased variance. When fitting the decay of the covariance, it is necessary to restrict the fitting to intervals greater than two dead times to avoid the distortion introduced by the filtering of the higher frequency components (Fig. 9 *C*).

Filter-induced errors in estimating the mean channel open time at low *Po*

When P_o is low, as is the case for very low concentrations of agonist for agonist activated channels, then the opening rate β in Eq. 6 approaches zero, and the mean channel open time for a two-state model can be approximated by the time constant of decay of the covariance. Because excessive filtering led to an overestimate of the time constant of decay of the covariance, estimates of mean channel open time obtained from the covariance were also overestimated (Fig. 9 *D*, P_o of 0.01). However, in comparison to estimates of i and N, estimates of mean channel open time were relatively insensitive to the effects of filtering as long as P_o was less than about 0.01–0.05. At such low P_o, errors in estimates of mean channel open time were less than about 10% provided that the cut-off frequency of the filter was greater than the dead time.

Channels typically open with bursts, rather than with single openings. If the shut intervals during the bursts are brief, then they can be missed with filtering so that burst duration becomes the effective mean open time (Fig. 6). Under such circumstances, which have often been the case for fluctuation analysis of agonists activated channels, it is mean burst duration that is usually estimated from the decay of the covariance. On this basis, the errors in estimates of i and N and also in mean burst duration would be somewhat less than predicted from the true duration of the mean open times (compare Figs. 4 and 6).

Estimating mean channel open time from covariance analysis of data obtained at higher P_o

Since it is often difficult, if not impossible, to obtain data at low P_o, it would be useful if there were a method for estimating mean channel open time from currents obtained at higher P_o. A plot of the observed time constant of the covariance versus mean current provides such a method. The projected intercept with the ordinate gives an estimate of the mean channel open time at the low concentration (and low P_o) limit (Eq. 10 and Fig. 9 *B*). Eq. 10 was derived assuming a single open state with a mean channel open time independent of open probability. A nonlinear relationship between plots of the time constant of decay of the covariance versus the mean current could indicate that the mean lifetime of the open state is not independent of P_o and/or that there are two or more open states (with different mean lifetimes) whose relative occupancy changes with P_o. Thus, plots such as Fig. 9 *B* would provide useful information toward developing kinetic schemes.

Even if a nonlinear relationship were observed between the time constant of decay of the covariance versus mean current, the projected intercept of such plots on the ordinate could still place some limits on the mean channel open time at the low concentration limit.

Bessel or Butterworth filters

Filter-induced errors in estimates of i and N with nonstationary fluctuation analysis were slightly less with a Butterworth than with a Bessel filter for the same level of filtering (Fig. 7; same cut-off frequency setting of each filter at the −3-dB level as shown in Fig. 1 *A*). It is not clear why this was the case, since the filtered response with the Butterworth filter to a step response rose more slowly due to the higher frequency attenuation of the Butterworth filter (Fig. 1). Perhaps the overshoot and ringing induced by the Butterworth filter compensated in some manner for the slower rise. Since the differences in errors were small for the two types of filters, the decision as to which filter to use will depend on what other types of analysis are to be applied to the same filtered current record.

Butterworth is the filter of choice for estimating mean open time using spectral analysis (frequency domain), since the Butterworth filter is better than the Bessel filter at preserving frequencies lower than the cut-off frequency (Figs. 1 and 10 *D*). In contrast, Bessel is the filter of choice for estimating mean open time using covariance analysis, since the ringing of the Butterworth filter can distort the decay of the covariance so that accurate estimates of the decay can not be obtained (Fig. 10, *A* and 10 *B*). Since Bessel filtering is preferred for single channel analysis (Fig. 1 *B*; Colquhoun and Sigworth (1983)), Bessel filtering and covariance analysis has the advantage over Butterworth filtering in that Bessel filtering allows the same current records to be used for the analysis of both current fluctuations and single channel records. Bessel filtering and covariance analysis also have the advantage of presenting the data in the same format as the probability density functions typically used for the analysis of single channel data. It can also be easier to visually resolve differences in single channel properties between two data sets presented as covariance functions rather than as spectral density functions (DeFelice, 1981, p. 221).

Practical considerations

The appropriate levels of filtering and the minimal durations of data required to prevent errors with nonstationary fluctuation analysis depend on the effective mean open time. Thus, the first step in preventing filter-induced errors is to estimate effective mean open time (burst duration in many instances). If it is not possible to obtain data at low P_o, or to verify that the data were obtained at low P_o, then mean open time should be estimated as shown in Fig. 9 *B*. Butterworth filtering should be used if mean open time is estimated by spectral analysis. Alternatively, Bessel filtering should be used if mean open time is estimated by covariance analysis. After determining effective mean open time, an appropriate level of filtering and the minimal durations of continuous current records to be analyzed can be selected for the nonstationary fluctuation analysis from the plots in Figs. 4, 6, and 8. To keep errors below 2–3%, the cut-off frequency of the low-pass filter should be greater than five times the inverse of the effective mean open time, and for continuous data segments the durations of the current records to be analyzed should be greater than 80 times the effective mean open time. With these conditions, which prevent excessive filtering, estimates of i and N could then be obtained with nonstationary fluctuation analysis using either Bessel or Butterworth filtering.

The two- and three-state models analyzed for this study are too simple to describe the detailed kinetics of most ion channels. Hence, the results of this study provide only some initial guidelines in assessing errors. Tests should always be performed to assure that the filtering is not excessive and that the durations of current analyzed are sufficiently long. Thus, repeated analysis of the data with progressively higher cut-off frequencies and increasing durations of current should be performed until the estimates of i and N no longer change. However, as indicated above, it must be remembered that excessive filtering may have already occurred during data acquisition, so that changing the final filter during the analysis may have little effect on the actual cut-off frequency of the effective filtering. Finally, once the channel parameters have been estimated using these guidelines, it would be worthwhile to simulate and analyze single channel currents with the estimated parameters at the same level of filtering used in the experimental analysis. A comparison of the estimated parameters from the experimental and simulated data for the model under consideration would then give a measure of the potential error in the analysis.

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