

SINGLE-CHANNEL CURRENTS FROM ACETYLCHOLINE RECEPTORS IN EMBRYONIC CHICK MUSCLE

Kinetic and Conductance Properties of Gaps Within Bursts

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ABSTRACT In tissue-cultured chick muscle, bursts of current from single nicotinic ion channels contain a variety of low-conductance gaps. One population has a lifetime of ~ 0.1 ms and an unknown conductance. A second population has a lifetime of 2–10 ms and conductance of zero. The third population has a lifetime of 0.5–1 ms and a mean conductance $\sim 2\%$ that of the main conductance state. This subconductance state has an agonist-dependent lifetime, longer for suberyldicholine than for acetylcholine, and is liganded to the same extent as the main conductance state. Subconductance gaps have a linear current-voltage behavior in the range -60 to -140 mV and appear to have the same reversal potential as the main state. The subconductance state is composed of a group of states which interconvert with correlation times longer than 300 μ s.

INTRODUCTION

When single-channel currents from nicotinic acetylcholine receptors were first observed by Neher and Sakmann (1976), the currents appeared as rectangular pulses of equal amplitude. With increases in the time and amplitude resolution of the patch-clamp technique (Hamill et al., 1981), the structure of the single-channel currents has been recognized to be more complex. Channel openings were observed to occur in clusters, or "bursts" (Nelson and Sachs, 1979). This bursting had been predicted by Colquhoun and Hawkes (1977) and suggested by Sakmann and Adams (1979) for a three-state kinetic model. A number of groups have analyzed nicotinic ion-channel activity using three- or four-state reactions (Colquhoun and Sakmann, 1981; Dionne and Leibowitz, 1982; Leibowitz and Dionne, 1983; Nelson and Sachs, 1981; Sine and Steinbach, 1983).

A more intricate picture of burst structure developed when Hamill and Sakmann (1981) noticed that nicotinic channels in tissue-cultured rat muscle could adopt multiple, discrete conductance states. The subconductance state (~ 10 pS) was easily observed at low temperatures, where it had a lifetime of several milliseconds. The reversal potential and selectivity of the subconductance state were indistinguishable from those of the main open state. Hamill and Sakmann also made the rather surprising observation that transitions between the subconductance state and the main state were not in equilibrium. Subconductance sojourns occurred following bursts, but not before bursts. In contrast to these results, Trautmann (1982) reported that transitions to and from the subconductance state appeared

to be in equilibrium, although his experiments were done with different agonists and at room temperature.

Auerbach and Sachs (1983) observed that in tissue-cultured chick muscle, gaps within bursts were heterogeneous, consisting of at least two conductance populations (0% and 10% of the open channel conductance) and another kinetically distinct population (time constant < 0.1 ms) of undetermined conductance. Because not all gaps have zero current, they cannot represent the closed, liganded state used in the kinetic analyses mentioned above.

In addition to showing a discrete subconductance state, Hamill and Sakmann's data (1981) showed substantial fluctuations of the subconductance current about its mean. These fluctuations are suggestive of additional states, perhaps similar to those reported by Sigworth (1981) for the main conducting state of the channel.

In the present study we have characterized the gaps within and adjacent to bursts in order to gain a physical understanding of what controls transitions to and from the various states within bursts. The results indicate that the subconductance state is actually an ensemble of states having an average conductance $\sim 12\%$ that of the main conductance state and a lifetime that depends on the agonist. There is a distinct population of relatively long-lived gaps whose mean current is effectively zero. Gaps within bursts do not involve ligand dissociation. The data show that transitions to and from the subconductance state are close to equilibrium.

METHODS

Pectoral muscles from 10–12 d chick embryos were dissected and dissociated in divalent cation-free saline containing 2 mg/ml Type IV

collagenase (Sigma Chemical Co., St. Louis, MO). The cells were counted in trypan blue and then plated in gelatin-coated 60-mm plastic culture dishes at a density of 10^6 cells/dish. The cells were grown in a tissue-culture medium containing Dulbecco's modified Eagle's medium (DMEM); 10% heat-inactivated horse serum (both from Grand Island Biological Supply, Grand Island, NY); 2% homemade embryo extract; penicillin; and streptomycin. Cells were incubated at 37°C in an atmosphere of 5% CO₂ and 95% air saturated with water vapor. After ~2 d in culture, the medium was changed to one containing 10^{-5} M cytosine arabinoside (Sigma) for 30 h to inhibit fibroblast growth. The experiments were performed on cells grown in culture 6–14 d. For recording, the growth medium was replaced with saline containing 140 mM NaCl, 4 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, and 10 mM Na HEPES (pH 7.4).

Patch pipettes were made from borosilicate glass capillaries and coated with Sylgard (Dow Corning, Midland, MI) according to Hamill et al. (1981). The patch amplifier was similar to that described by Hamill et al., except that we used a Burr-Brown OPA-101 amplifier for the current-to-voltage converter. The 3 db bandwidth of the system was >10 kHz. All experiments were done on cell-attached patches. The agonists were acetylcholine (ACh), 50–100 nM, and suberyldicholine (SubCh), 5–10 nM. The temperature was 22–24°C.

Data were recorded on analog magnetic tape and then transferred to digital tape at a sampling rate of 20 kHz. The anti-aliasing filter was a four-pole Bessel set at 8 kHz. The bandwidth was further limited by a finite-impulse-response (FIR) digital filter which had a Gaussian response in both the time and frequency domains (Colquhoun and Sigworth, 1983). The effective bandwidth of our analyses ranged from 2–6 kHz. The data were analyzed by an automated pattern-recognition program (Sachs et al., 1982) which utilized a half-amplitude threshold-crossing criterion to identify transitions and a pattern-matching algorithm to reject noise. The baseline estimate was updated approximately every 25 ms. We arbitrarily defined bursts as a series of channel openings separated by gaps shorter than 7 ms.

To measure amplitudes accurately, we had to reject data that included the effects of amplifier-filter settling. We rejected all data within a "dead time" preceding and following each transition. This dead time was set equal to $0.6/f_c$, where f_c is the 3-db bandwidth. Thus, at 5 kHz our dead time allowance was three samples (150 μ s) and our total allowance for settling and delays was 300 μ s, so that we analysed only gaps that were 350 μ s or longer. The half-amplitude method for measuring event durations is biased for events that are less than $\sim 0.5/f_c$ (Sachs and Auerbach, 1983). Therefore, when fitting histograms of event durations, we imposed a dead-time allowance similar to that used for amplitude analysis.

We estimated event amplitudes in two ways. First, each data point (exclusive of the settling/delay allowance) was entered into an amplitude histogram which we will call a total-current histogram. Second, we computed the mean of the data points within each gap and stored this value with the corresponding gap duration. From these data we compiled histograms of mean amplitude and/or duration.

Curve fitting was done with a general-purpose nonlinear least-squares algorithm. Histograms were fit to the integral of the appropriate probability density function evaluated over each bin. This allowed for variable bin widths and prevented the finite bin width from biasing the fit. To allow for the Poisson distribution of counts in each bin, the squared residuals were weighted inversely with the number of counts in each bin (Bevington, 1969). To avoid biasing the fit when the histogram was sparse, bin widths were adjusted so that each bin contained at least five counts (Sachs and Auerbach, 1983). For signal-averaged transitions (see below) the data points were equally weighted. Except where otherwise noted, confidence limits are "support plane" estimates at the 90% level (Marquardt, 1964). These are symmetrical confidence limits on a parameter independent of the errors in the other parameters.

Histograms of mean gap amplitude presented a special problem because the variance of each measurement depended upon the event duration. Amplitudes of long events were more precise than those of short

events. For these data the sample variance was inversely proportional to the number of data points in the average. Therefore, for these data, in addition to the standard histogram weighting procedure, the residuals were weighted in proportion to the number of raw data points contributing to each bin. Thus weighted, the variance of a Gaussian fitted to the mean amplitude distribution is dominated by the variance of the underlying population and not the measurement errors. This population variance included a component from intrinsic variability of the conductance and a component from errors in the baseline estimation.

We wanted to estimate the kinetics of subconductance gaps separately from zero current gaps. We fit the amplitude-duration data to a statistical model where the mean amplitude of each event was assumed to be drawn from one of two Gaussian distributions and where the duration of events in each amplitude distribution was described by an exponential probability density function. The variance of the amplitude distribution was taken to be a sum of the population variance (σ_p^2) and the single-sample, wideband variance (σ_s^2) normalized by the number of data points used to calculate the mean amplitude of the event (N),

$$\sigma^2 = \sigma_p^2 + \sigma_s^2/N. \quad (1)$$

Thus, the joint probability density of obtaining an event of amplitude a and duration d was given by

$$P(a,d) = k \exp(-kd) \times (1/\sigma\sqrt{\pi}) \exp\{-0.5([a - \langle a \rangle]/\sigma)^2\} \quad (2)$$

where $\langle a \rangle$ has been taken from Eq. 1 above, $\langle a \rangle$ is the mean of the appropriate amplitude distribution, and k is the mean of the appropriate duration distribution. In practice, the time distribution has to be normalized to cover the actual range of the data, rather than the usual range from zero to infinity. The relevant distribution means and variances were estimated as described above.

We then used the maximum likelihood technique to fit the two time constants. The likelihood of observing a particular set of N events is

$$L = \prod_{i=1}^N P(x_i),$$

where $P(x_i)$ is the probability of observing event x_i (Bevington, 1969). L , actually $\log L$, was maximized with respect to the parameters by using a Newton-type algorithm. We tested that the suspected maximum was indeed global by generating multiple random starting points. Approximate confidence limits were obtained by holding one parameter constant and then changing the other until the likelihood fell to the requisite level. Thus, a 90% confidence interval would correspond to a set of parameters that make the data set 10% as likely.

To estimate the likelihood of passage through a subconductance state preceding and following bursts, we aligned the leading and trailing edges of bursts at the moment of threshold crossing and analysed the average current. When subconductance sojourns are present, these burst transitions are preceded and followed by exponential phases that reflect the lifetime of the subconductance sojourn. The amplitude of the decay (or rise) extrapolated to the moment of threshold crossing is the product of the amplitude of the subconductance current and the probability of passing through a subconductance state. We curve fit the average current tails to single exponentials with an offset to allow for slight differences in the estimation of baselines before and after bursts.

RESULTS

Kinetic Characteristics of Gaps Within Bursts

Fig. 1 shows several bursts of current from channels activated by acetylcholine in which subconductance gaps

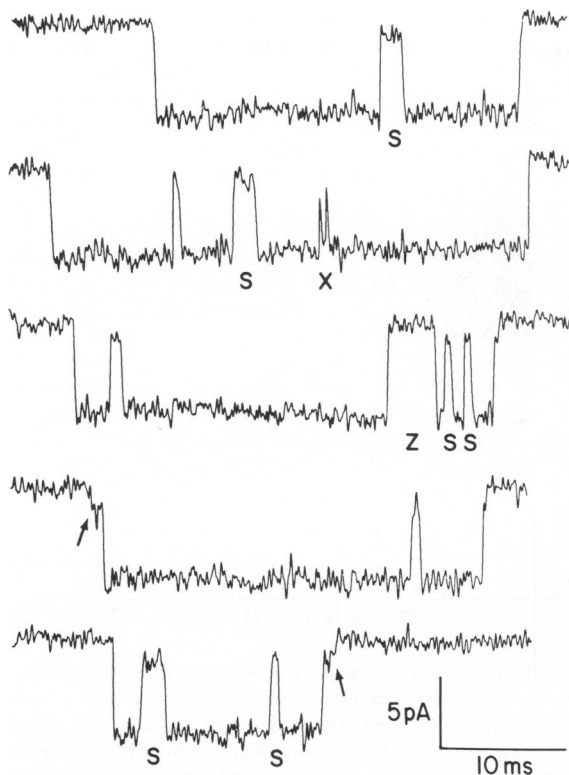


FIGURE 1 Examples of gaps within bursts of nicotinic channel currents. The gaps fall into one of three categories: those with zero current (*Z*), those with nonzero current (*S* for subconductance), and those too short to have amplitudes that could be determined (*X*). Most of the burst edges rise and fall cleanly from the baseline. In the lower two traces, subconductance sojourns on the leading and trailing edges of bursts are marked with arrows. The agonist was 50 nM ACh, the pipette was held at 80 mV relative to the bath, and the data were analyzed at a bandwidth of 3.0 kHz.

are clearly visible. Isolated examples such as these cannot be taken as proof of subconductance sojourns because the overlap of gap current with baseline noise or with independent channel currents can produce gaps having nonzero current. A statistical analysis of gap amplitudes shows, however, that a large percentage of the gaps that appear during bursts do have a current greater than zero (Auerbach and Sachs, 1983). The result is graphically illustrated in Fig. 2, where we have aligned and averaged the edges of several hundred gaps within and between bursts. The mean amplitude of the current step was smaller for gaps within bursts than for gaps between bursts. The transition times are similar to the calibration pulse (dotted line). In the record shown, the fraction of bursts that terminated through a subconductance state was small, because the average burst trailing edge closely resembles the response of the amplifier.

As an independent test of our methods of analysis, we examined the amplitude characteristics of gaps produced by the local anesthetic QX-222 in nicotinic channels, and gaps present in Ca^{++} -activated K^{+} -channel currents (Fig.

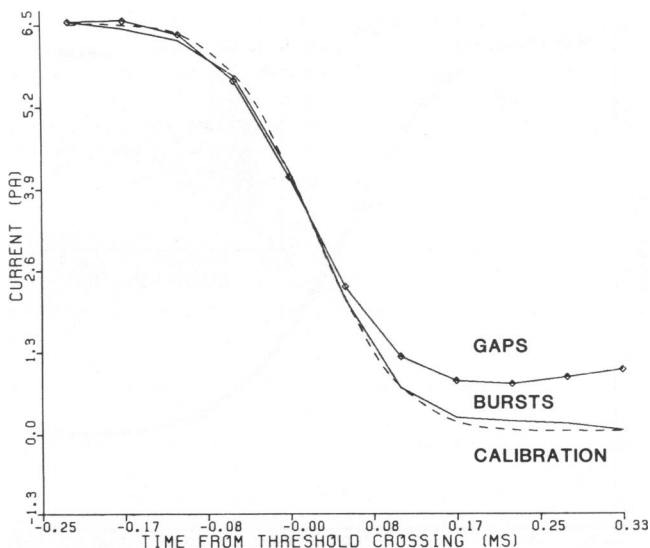


FIGURE 2 A comparison of the mean closing-transition amplitudes of gaps within bursts (connected symbols) and burst trailing edges (solid line, —). Inward current is shown upward. For reference, a calibration trace from a capacitively-coupled triangle wave is included (dashed line, ---). The appropriate transitions were aligned at the moment of threshold crossing and were signal-averaged. Only intraburst gaps longer than 450 μs were included in the average, accounting for the rather flat mean gap amplitude. The transition amplitude of gaps within bursts is less than that of burst terminations (gaps between bursts). Analysis of the distribution of gap mean amplitudes (cf., Fig. 5) showed that in this record, over 90% of the gaps within bursts were from a subconductance population. The experimental conditions are the same as Fig. 1.

3). The amplitude of virtually all gaps from both types of channel was zero.

Our methods for estimating the amplitudes and durations of conductance events are illustrated in Figs. 4–6 for one sample data set. The channels were activated by 50 nM ACh. Recall that in these figures, event amplitudes were measured only after allowing for amplifier and filter settling.

Fig. 4 shows a scatter plot of mean gap amplitude vs. duration for 483 events, 0.35–7.0 ms in length. Two amplitude distributions are visible, one close to zero and the other near 1.5 pA. Although not shown, a similar scatter plot of the main open channel amplitude vs. duration showed only a single distribution.

The two components of the amplitude distribution are shown more clearly in Fig. 5, where the mean gap amplitudes have been compiled into a histogram. The means, based on a sum of Gaussians fit to the histogram, were 0.01 ± 0.07 pA and 1.28 ± 0.12 pA. A similar analysis of the open channel scatter plots yielded a unitary current of 6.23 ± 0.01 pA. The standard deviation of the subconductance population (0.49 ± 0.15 pA) was significantly greater than that of the zero current population (0.23 ± 0.08 pA). From the areas under each distribution, 75% of the events were from the subconductance population.

We also estimated the mean currents of the two popula-

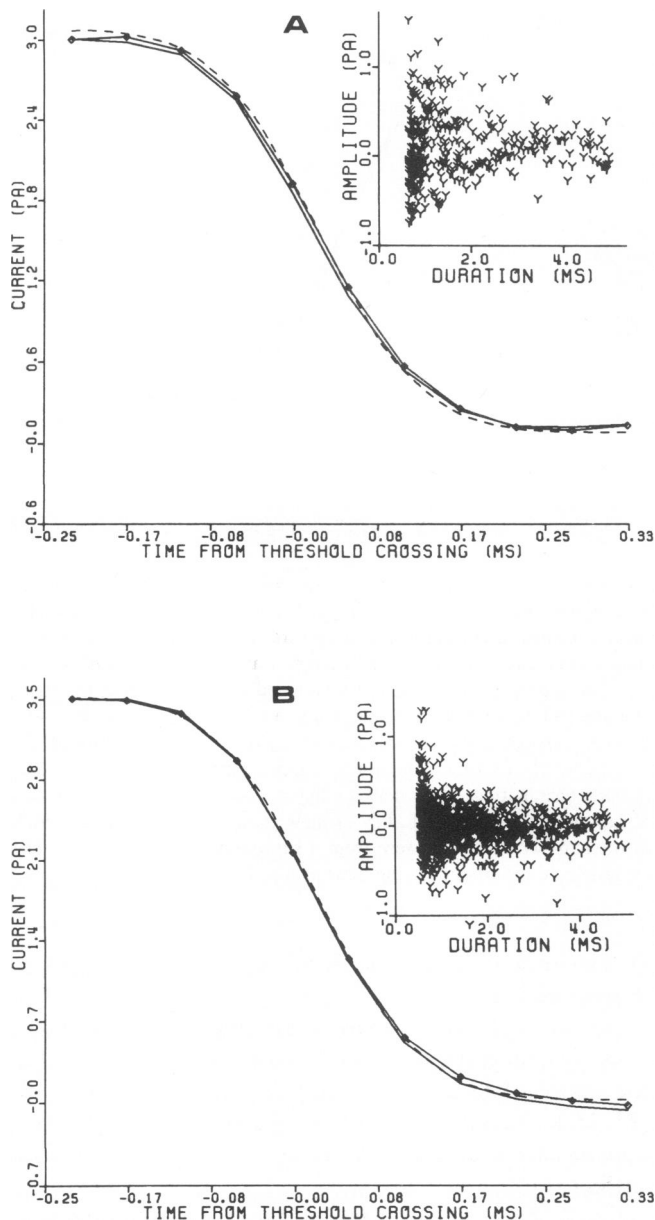


FIGURE 3 Mean closing transitions of gaps within and between bursts from nicotinic channels blocked by QX-222 (*A*, inward current up) and from Ca^{++} channels (*B*, outward current up). The notation is the same as in Fig. 1. The insets show the mean amplitude/duration cross correlations for gaps 0.6 ms or longer. In both experiments, virtually all of the gaps within bursts had zero current. In the QX-222 experiment (*A*), the pipette contained 10 nM SubCh and 20 μM QX-222. In the K^+ channel experiment (*B*), the pipette contained normal saline only and was held at -60 mV relative to the bath. The bandwidth of the analyses was 2.0 kHz.

tions from the amplitude histogram of all data points within gaps (Fig. 6). The data points of this distribution are equally weighted. Fitting this total-current histogram with the sum of two Gaussians yielded means for the zero current and subconductance population at -0.09 ± 0.05 pA and 1.43 ± 0.08 pA. From the areas under each distribution we estimated that only 39% of the data points were contributed by subconductance gaps.

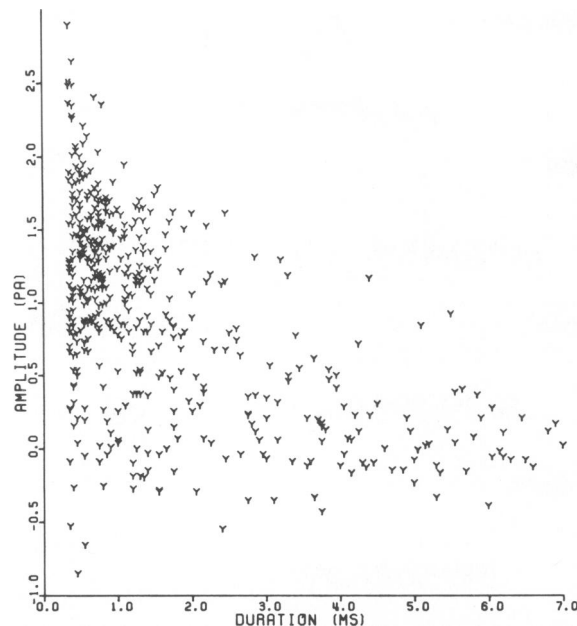


FIGURE 4 Plot of mean amplitude vs. duration of gaps within bursts. There are two clusters of events, one with an amplitude near 1.5 pA and with an amplitude near 0 pA. The zero current gaps appear to last longer than the subconductance gaps. The agonist was 50 nM ACh, the pipette was held at $+80$ mV relative to the bath, and the analysis bandwidth was of 5.0 kHz.

The observation that a majority of the events but a minority of the data points came from subconductance gaps is evidence that zero-current gaps are longer than subconductance gaps. This difference in duration can be seen in the scatter plot of Fig. 4, where at long times there are proportionally fewer events of the subconductance

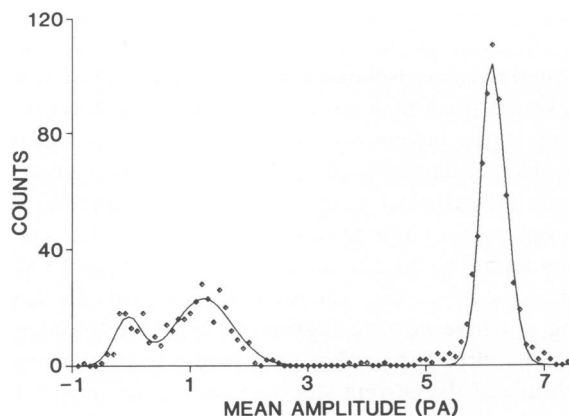


FIGURE 5 Histogram of the mean amplitudes of events within bursts. Three distinct populations can be distinguished. Openings to the main conductance state had an average current of 6.2 pA, subconductance gaps had an average current of 1.3 pA, and zero current gaps had an average current of 0.0 pA. Of the low conductance events, an estimated 75% were from the subconductance population. The data for events whose amplitude was less than half the open channel current were compiled from the scatter plot shown in Fig. 4. The data for the main conductance state came from a similar analysis. Note that the distribution variance is greatest for the subconductance population. The bandwidth of the analysis was 3.0 kHz.

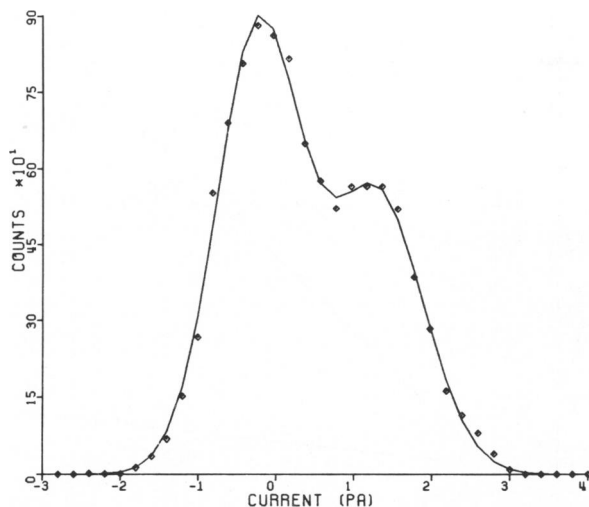


FIGURE 6 Histogram of the amplitude of data points within gaps. The effects of amplifier and filter settling were eliminated by excluding data points preceding and following transitions. Two populations are apparent, one near 1.4 pA and the other near 0 pA. 39% of the data points belong to the higher-current population. The data are from the same record as Fig. 4. The bandwidth was 3.0 kHz.

amplitude. We used the maximum-likelihood technique described in Methods to quantify the kinetic difference between the subconductance and zero current populations. For simplicity, we assumed that the lifetime of each amplitude distribution could be described by a single exponential. Using the amplitude means and variances described above as fixed parameters, and fitting the two time constants, we found that for the experiment shown in Fig. 4 the subconductance population had a lifetime of 0.66 ± 0.14 ms while the zero-current population had a lifetime of $6.45 \pm 13.5 / -3.2$ ms. The wide confidence limits on the time constant of the zero-current population reflect the fact that few of the zero-current events occurred within the 7 ms closed-time limit we used to define a burst.

These time constants were compared with the time constants estimated from an unconditional histogram of closed times. In the range 0.5 to 7.0 ms the data were described by the sum of two exponentials. For the experiment shown in Fig. 7, the time constant of the faster process was 0.58 ± 0.16 ms and that of the slower process was 6.85 ± 2.02 ms, with an estimated 77% of the events belonging to the short time-constant population. Thus, there is an excellent agreement between the results from the maximum likelihood analysis and the unconditional distribution. This agreement supports our assumption that there is only a single kinetic component to the population of subconductance gaps.

Could the zero current gaps be associated with interburst closed periods, i.e., those times which were likely to have included sojourns to an unliganded state? A histogram of durations longer than 20 ms is shown in the *inset* of Fig. 7. The time constant of the slow component was 340 ms (109 events). We expect only two events from this

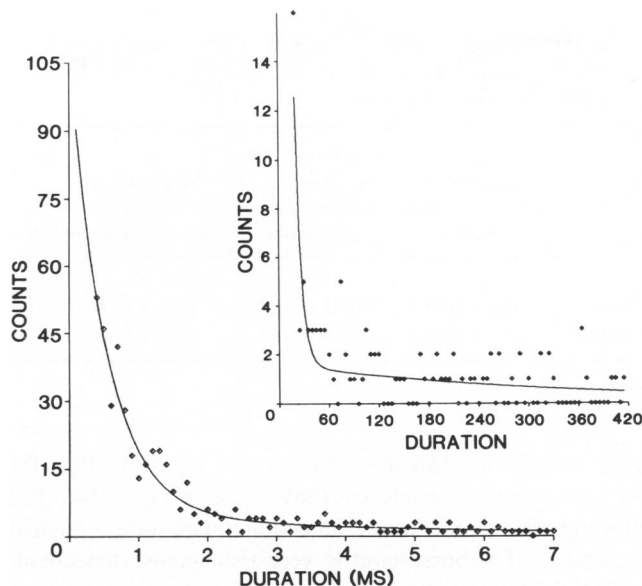


FIGURE 7 Histogram of the durations of gaps within bursts. The data were compiled from the scatter plot shown in Fig. 4. In the range 0.45 to 7.0 ms, the data were fit to the sum of two exponentials with characteristic time constants of 0.5 and 6.9 ms (—). The maximum likelihood method of determining the subconductance population lifetime gave an estimate of 0.6 ms, suggesting that there is a close correlation of the substate lifetime with the shorter of the two time constants measured from the unconditional distribution of gap durations. The *inset* shows the distribution of long closed periods (primarily of gaps between bursts) fit to a sum of two exponentials (—). The shorter time constant component corresponds to the 6.9 ms component of intraburst gaps. The time constant of the longer component was 340 ms.

distribution shorter than 7 ms, while we measured over 100 such zero current gaps. Thus, we can say that the zero current gaps within bursts are not from the same distribution as are the long, interburst closed periods. Our estimates of the lifetime of gaps with zero current varied but remained in the range of 2–10 μ s.

In addition to the two intraburst closed periods described above there was a third component of very short gaps which had a lifetime of ~ 0.1 ms. Because the lifetime of this population was too short to permit precise amplitude measures, we have no information about whether this component represented transitions to a subconductance or to a zero current state. The fraction of all gaps belonging to this population was quite variable, accounting for <10% to >80% of all gaps within bursts. This variability was consequently reflected in the channel open time. These brief gaps may represent sojourns to a doubly-liganded, closed state as per Colquhoun and Sakmann (1981); transient blockage by a diffusible substance present at concentrations $>10 \mu$ M; or simply another short-lived conformational state with a low conductance.

We used the maximum-likelihood method to examine the agonist dependence of the lifetime of subconductance gaps. The results for ACh and SubCh are described in Table I. For SubCh-activated channels the lifetime was

TABLE I
AGONIST DEPENDENCE OF THE AMPLITUDE
AND KINETIC PROPERTIES OF SUBCONDUCTANCE
GAPS WITHIN BURSTS

	Lifetime of subconductance gaps	Fraction of gaps from subconductance population	Amplitude as a fraction of main state current	N
	ms			
ACh	0.61 ± 0.25	0.84 ± 0.20	0.11 ± 0.09	10
SubCh	1.13 ± 0.28	0.79 ± 0.16	0.12 ± 0.04	12

(mean \pm SD).

1.13 ± 0.28 ms (mean \pm SD, $n = 12$), while that for ACh-activated channels was 0.61 ± 0.25 ms ($n = 10$). The lifetime of a channel in the subconductance state is agonist dependent. The burst lengths were also agonist dependent, with SubCh bursts longer than ACh bursts.

We find that in tissue-cultured embryonic chick pectoral muscle there are at least two types of nicotinic channel which can be distinguished on the basis of main open state conductance. One type has a conductance of 50 pS and the other type a conductance of 35 pS. This observation parallels those from other tissue-cultured systems (cf. Hamill and Sakmann, 1981; Trautmann, 1982). To test whether the lifetime of subconductance gaps was different for the two channel types, we split the data set for ACh-activated channels according to the main state conductances. For the 50-pS channel, the gap lifetime was 0.59 ± 0.12 ms ($n = 4$) and for the 35-pS channel the gap lifetime was 0.62 ± 0.32 ms ($n = 6$). The subconductance-state lifetime appears to be the same in both channel types.

The subconductance-state lifetime also did not depend on the membrane potential over the range of -66 to -140 mV.

Is there an agonist dependence to the probability of a channel entering a subconductance (as opposed to a zero current) state within a burst? We compared the relative areas under the two peaks fit to the gap mean amplitude histogram (cf., Fig. 5) for ACh- and SubCh-activated channels. The results are shown in Table I. There was substantial scatter in the data from different patches. For 35-pS channels activated by SubCh, the fraction of subconductance gaps ranged from 0.43 to 0.97. Over all experiments, regardless of agonist, channel conductance, or voltage, the fraction of subconductance gaps was 0.81 ± 0.18 (mean \pm SD), but with no clear indication of agonist or voltage dependence to this value.

Amplitude Characteristics of Gaps Within Bursts

Fig. 8 shows the current-voltage (I - V) relationship for both subconductance and main conductance states of SubCh-activated channels. Within the accuracy limits of the

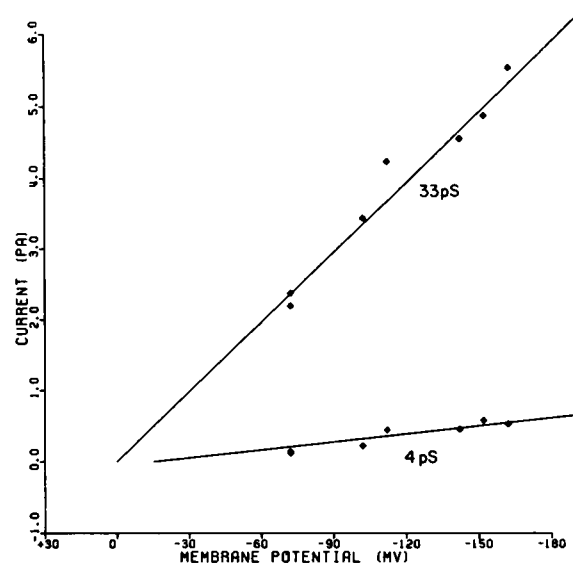


FIGURE 8 Current-voltage relationship of the open channel and of subconductance gaps. The estimates of event current were obtained by fitting mean amplitude histograms to Gaussians (see Figs. 4 and 5). The estimates of membrane potential were obtained by assuming a zero-current potential of 0 mV for the main conductance state. The current-voltage relationship was fit to a straight line with the residuals weighted inversely with the 90% confidence limits on the current amplitude estimates. The conductance of the main open state was 33 pS and that of the subconductance state was 4 pS. The extrapolated reversal potential of the subconductance state was 15 mV hyperpolarized to that of the main conductance state, and the estimated cell resting potential was 72 mV. The data came from three separate patches from the same region of a single cell. The agonist was 5 nM SubCh.

technique, both classes of event showed a linear I - V relationship, corresponding to conductances of 4 and 33 pS. The extrapolated reversal potentials for the main and the subconductance state were 0 mV and -15 mV, assuming a resting potential of -72 mV. This difference of 15 mV was not statistically significant. In many patches we could not obtain sufficient data to determine the I - V relationship precisely, so we chose to express the subconductance gap current as a fraction of the unitary current. Over all experiments, the subconductance gap current was $12 \pm 5\%$ of the unitary current. The ratio of substate to main state current ranged from 0.06 to 0.23, with no apparent agonist dependence (Table I).

Current Fluctuations of Events Within Bursts

As mentioned above, when the histogram of mean gap amplitude is fit to a sum of Gaussians, the variance of the subconductance component is substantially greater than that of the zero current component. This observation suggests that the subconductance state is in fact an ensemble of states. To estimate the spectral characteristics of this excess variance, we measured variances of events at five different bandwidths. For example, in the experiment corresponding to Fig. 5 (3 kHz bandwidth), the variances

were baseline (gaps between bursts), 0.38 pA^2 ; main open channel current, 0.53 pA^2 ; subconductance gaps, 0.57 pA^2 ; and zero-current gaps, 0.45 pA^2 . In Fig. 9 we have plotted the excess variance (signal variance minus baseline variance) for the open channel, the subconductance gaps and the zero current gaps as a function of the data bandwidth. For reference, Fig. 9 includes the Lorentzian distribution function given by a \tan^{-1} function). The distribution function rises to a maximum, equal to the total variance, reaching half maximum at the cutoff (3db) frequency.

The subconductance gap fluctuations are rather long lived, compared to those of open channels, persisting for mean times greater than 0.3 ms. These data are consistent with the results of Hamill and Sakmann (1981), which showed increased noise of the substate relative to both the fully closed and open states.

Equilibrium Properties of Bursts

Hamill and Sakmann (1981) proposed that transitions between the main state and the subconductance state are not in equilibrium. They reported that the probability of a substate preceding a burst is much less than the probability of a substate following a burst. As one measure of equilibrium, we aligned and averaged the leading and trailing edges of bursts. In this form, subconductance sojourns preceding

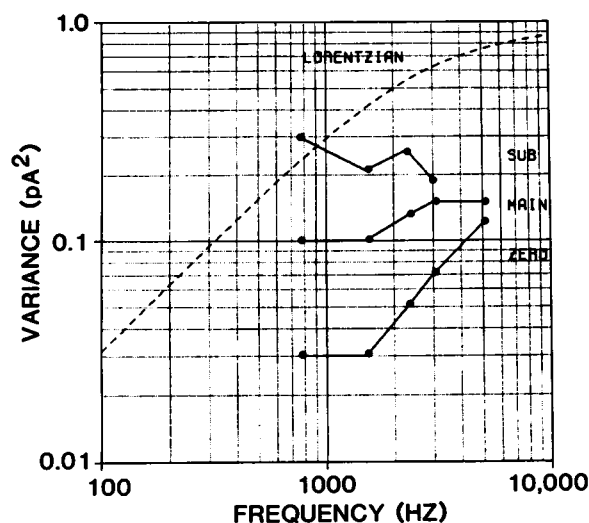


FIGURE 9 The spectral distribution function of the current fluctuations of events within bursts. Each curve represents the integral of the variance up to the frequency indicated. The dotted line (---) shows what a Lorentzian power spectrum looks like in this format (cutoff frequency at 5 kHz). The *SUB* trace represents fluctuations in subconductance gaps, whose mean current in this experiment was $\sim 1.3 \text{ pA}$. The *MAIN* trace represents the fluctuations of the main open state, whose amplitude was $\sim 6.2 \text{ pA}$. The plot indicates a cutoff at $\sim 2.5 \text{ kHz}$ for the main open state, which is the same as that observed from conventional power spectra (Auerbach and Sachs, unpublished observations). The *ZERO* trace represents the fluctuations of those gaps within bursts that had a mean current close to zero. The most pronounced feature of this trace is the strong frequency dependence suggestive of opening processes with durations shorter than $50 \mu\text{s}$ (taken from the same record as Fig. 4).

bursts appeared as an exponential buildup of current, while those following bursts appeared as an exponential decay. An example of such an analysis is shown in Fig. 10. Fitting an exponential to the falling phase of the burst trailing edge gave an estimate of a process with a time constant of $0.60 \pm 0.20 \text{ ms}$. A similar fit to the leading edge of the burst (shown time-reversed in Fig. 10) gave a time constant estimate of $0.55 \pm 0.15 \text{ ms}$. In the same record, the lifetime of subconductance gaps was 1.08 ms . The intercepts of the exponential phases with the point of threshold crossing is the product of the subconductance current and the probability of passing through the subconductance state. For the experiment shown in Fig. 10, the intercept of the rising phase was $0.6 \pm 0.1 \text{ pA}$ and that for the falling phase was $0.5 \pm 0.1 \text{ pA}$. The substate current derived from the histogram of mean gap amplitudes was $0.40 \pm 0.03 \text{ pA}$. Assuming that the current amplitudes of inter- and intraburst substates are equal virtually all bursts began and ended with subconductance sojourns. For comparison, in this record, over 90% of all gaps within bursts were to a subconductance state.

Compared with intraburst substate gaps, the probability of occurrence of substates on burst edges was highly

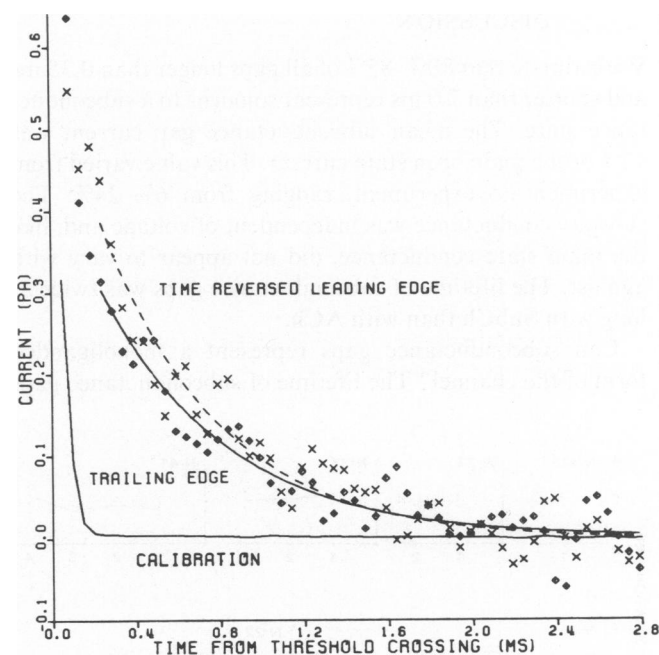


FIGURE 10 Signal-averaged leading and trailing edges bursts. When substantial numbers of subconductance sojourns are present before and after bursts, exponential phases of current precede and follow the mean burst transitions. The open channel current was 3.83 pA , far off scale. The averaged trailing edges (\diamond and —) and leading edges (\times and ---, shown time-reversed) were each fit to a single exponential from $350 \mu\text{s}$ to 2.8 ms after (or before) crossing half amplitude. The amplifier/filter response to a capacity coupled triangle wave is shown in the trace labeled *CALIBRATION*. The fitted curves are similar with regard to time constant and area, indicating that the forward and reverse rates are close to being in equilibrium. As an index of variability, compare these results with those shown in Fig. 2 where few subconductance sojourns were observed on burst edges. The agonist was 5 nM SubCh.

variable. Some records showed virtually no exponential phase either preceding or following bursts (compare Figs. 2 and 10). There was a tendency for the exponential phases of current preceding and following bursts to be faster, by about a factor of two, than the lifetime of intraburst substate gaps. Nonetheless, when exponentials were clearly visible, the time constants and intercepts of the currents before and after bursts did not differ by more than a factor of two, with no trend as to which was larger. Transitions to and from the subconductance state appear to be close to equilibrium.

If transitions into bursts were out of equilibrium, we might expect that the open states would be out of equilibrium. To test this, we measured the duration of events in bursts as a function of position within the burst. Symmetric variations in this parameter are kinetically interesting but do not indicate lack of equilibrium (Colquhoun and Hawkes, 1982), while asymmetric variations do indicate a lack of equilibrium. Fig. 11 shows the results for one record in which there were a large number of events so that the standard deviations of the durations were small. In all records there was no correlation, either symmetric or asymmetric, of the open time with position in the burst.

DISCUSSION

We estimate that 80%–85% of all gaps longer than 0.35 ms and shorter than 7.0 ms represent sojourns to a subconductance state. The mean subconductance gap current was 12% of the main open state current. This value varied from experiment to experiment, ranging from 6%–24%. The substate conductance was independent of voltage and, like the main state conductance, did not appear to vary with agonist. The lifetime of subconductance gaps was twice as long with SubCh than with ACh.

Can subconductance gaps represent a monoligated form of the channel? The lifetime of subconductance gaps

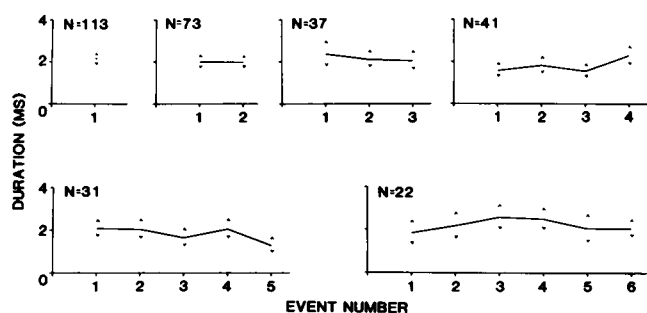


FIGURE 11 The mean duration of channel open times as a function of position with bursts. All bursts of 1, 2, 3...etc. events were separately collected and the durations of corresponding events were averaged and plotted as a function of position within the burst. The standard deviations for each mean are indicated by the arrows. The number of bursts contributing to each mean are indicated in the figure. The fact that there is no asymmetry in the durations suggests that the channel closing rates are in equilibrium throughout a burst. The experimental conditions were the same as in Fig. 4.

for SubCh-activated channels was ~ 1 ms. Assuming a diffusion-controlled binding rate of $10^9 \text{ s}^{-1} \text{ M}^{-1}$, and an agonist concentration of 10 nM, the rate of gaining a ligand would be 10^1 s^{-1} , equivalent to a lifetime of 100 ms. Thus the subconductance state must be liganded to the same extent as the main open state. The same argument holds for the other classes of gaps within-bursts.

The subconductance state appears to be heterogeneous, as the variance of the subconductance gap current is consistently larger than the current variances of the baseline, the zero current gaps, and the main conductance state. Furthermore, the excess variance (over the baseline) did not vary with bandwidth in the range from 0.75–3.0 kHz. (The frequency range was limited on the low end because few events persisted long enough to permit filter settling, and on the high end because noise resulted in threshold crossings which prematurely terminated the gaps).

Can a two-state model account for the excess variance? Suppose that within a subconductance gap there are sojourns to zero-current (gaps within gaps). The mean ($\langle I \rangle$) and variance (σ^2) of the subconductance gap current would be given by: $\langle I \rangle = ap$, $\sigma^2 = a^2p(1 - p)$, where a is the true subconductance current amplitude and p is the probability that during a gap the channel carries current a (Lecar and Sachs, 1981). In the example shown in Figs. 4–7 the amplitude of the subconductance current was 1.28 pA and the excess variance was $\sim 0.25 \text{ pA}^2$. Solving two equations above we found that $a = 1.43 \text{ pA}$ and $p = 0.90$.

For a simple two-state system with opening rate k_o and closing rate k_c , $p = k_o/(k_o + k_c)$, $\tau = 1/(k_o + k_c)$, where τ is the relaxation time constant. Because the variance was constant over the range 0.75–3.0 kHz, $\tau > 320 \mu\text{s}$, $k_o < 2.8 \times 10^3 \text{ s}^{-1}$ and $k_c < 0.3 \times 10^3 \text{ s}^{-1}$. This means that the excess variance could arise if the true subconductance current were 1.43 pA and if it were interrupted by complete closures longer than $360 \mu\text{s}$ at intervals longer than 3 ms. In the experiment corresponding to the above calculation, the subconductance gap lifetime was 0.6 ms. Thus, most subconductance events would not contain a closure and should therefore have a variance approximately equal to that of the baseline. In fact, the population variance of subconductance gaps appeared to be independent of event duration (Fig. 9). We therefore conclude that a simple two-state model of gaps within gaps is wrong and that there are more than two components to the substate. The mechanism that accounts for the excess fluctuations in the substate current may also account for the fluctuations in the open channel current (Sigworth, 1981, and above). More complete analyses of spectra are necessary to determine whether fluctuations arise from current variations within each gap or from heterogeneity between gaps.

As with subconductance gaps, the variance of the zero-current gaps is greater than the baseline. The result indicates that the mean current cannot actually be zero, although it is indistinguishable from zero in our experi-

ments. Because the spectral distribution function for zero-current gaps (Fig. 9) does not level off by 5 kHz, these fluctuations must arise from a process, or processes, having correlation times $<30 \mu\text{s}$. The excess variance might arise from openings which are too brief to reach the detection threshold. At 5 kHz, events shorter than $\sim 40 \mu\text{s}$ do not reach threshold (Sachs and Auerbach, 1983). The two-state analysis used above predicts that the observed excess variance could be explained by full channel openings shorter than $30 \mu\text{s}$ occurring at intervals of 1.9 ms. These kinetics predict a mean current of 0.1 pA, close to the accuracy limits of our experiments.

The drop in conductance associated with the subconductance state implies a change in the rate-limiting step(s) to permeation. Because the subconductance state current and the reversal potential follow the main state, the change in permeation rates does not appear to involve a change in a single energy barrier (Hille, 1975). In our experiments, the I - V relationship of subconductance gaps was linear. If a single barrier were to become rate limiting, the I - V relationship would be expected to approach an exponential, although it is not clear how much deviation from linearity would be apparent over the range of voltages we have used. A simple phenomenological interpretation of the subconductance state is that a well in the free energy profile for permeation is transiently deepened, producing a fivefold drop in conductance but no change in selectivity. It is intriguing that both the lifetime of the subconductance state and the burst length are similarly influenced by the agonist, suggesting that the ion-binding site and the gate may be identical.

Like Trautmann (1982), we found no evidence that either the main conductance or the subconductance states of the channel are far from equilibrium. Our measurements showed that within a factor of two, forward and backward reactions occur with equal frequency. We have observed deviations greater than the apparent experimental error, but not in a consistent direction. In no case did the deviations approach the level of 2^{39} reported by Hamill and Sakmann (1981). We have no explanation for the difference in results, with the possible exception that the temperature in Hamill and Sakmann's experiments was significantly lower (8°C) than either ours or Trautmann's. It would be surprising, however, to find such a dramatic temperature dependence to the reaction unless there were some form of phase transition.

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DISCUSSION

Session Chairman: Harold Lecar *Scribes:* William McCarthy, Joshua Zimmerberg, and Michael Kell

HORN: Given data like that reported in your paper, what do you think is the relevance of the usual chemical kinetic schemes that we are all making?

AUERBACH: We very carefully avoided drawing models. We know that all of the subconductance states cannot be identical; those in the center of the burst seem to be longer lived than those on the edge. The lifetime of the substate is dependent on which agonist is used, being about twice as long for suberyldicholine as it is for acetylcholine. The simplest model that we can think of is one where the channel becomes doubly liganded (A_2R), enters a group of six states which can interconvert freely, and then closes. This group of states includes the main, subconductance, and zero conductance states. Gaining a physical interpretation of what is underlying, these states would be interesting; however, the interpretation of a global model describing the interactions of these states, even using the maximum likelihood method, would be difficult.

DURKIN: You have two distinct conductance classes, 35 and 50 pS. Hamill and Sakmann have also seen this in embryonic chick muscle, and Alain Trautman has seen this in rat myotubes. Because these are all immature cell types, perhaps you are seeing molecularly distinct isoforms. Do you have any data on this? Can you perform any conditional probability tests to determine their possible independence?

AUERBACH: In chick muscle there is very little difference between the 35 and 50 pS channel burst length, just as there is very little difference between the kinetics of junctional and extrajunctional nicotinic channels in adult chicken muscle. We don't believe that the amplitude of the subconductance state (~12% that of the main conductance state) depends on the amplitude of the main conductance state. We haven't done any correlation tests, but we don't see a channel switching from 35 to 50 pS states within a burst; the amplitude stays fairly constant. In our measurements on the frequency dependence of the excess variance we saw a possible DC component, which could indicate that the base state and each of the levels of the substates could be composed of a group of states having very small differences in conductance.

PATLAK: You implied that the presence of subconductance states is variable. If this state is not present in a particular channel, does the intermediate closed time component also drop out, or does the conductance of that subconductance state becomes zero?

AUERBACH: We have extracted time constants for individual events within bursts for only a handful of data sets; we don't have enough data yet to obtain a meaningful relation by correlating time constant and amplitude. As for variability, some conductance states exhibit considerable variability as to the appearance of substates at the edge of a burst, the number of ultrafast glitches within a burst, and the amplitude of the subconductance states. What does not seem to be variable is the duration and relative frequency of occurrence of the subconductance states.

DIONNE: Some of your data show evidence of temporal complexities and amplitude sublevels, and some data less. Have you identified any variables, for example, culture conditions, ionic constituents, or temperature, which seem to correlate with the appearance of these complexities?

AUERBACH: No, we have not.

GUY: Have you determined whether the frequency of an event is a function of its position within the burst? Do short or low conductance events occur more frequently at the beginning or end of the burst?

AUERBACH: We have plotted the mean duration of the event within a burst as a function of position within the burst, and we find no significant trend. We do not know whether the distribution of durations as a function of position are the same; all we know is that the mean is not different. Because we can identify five of the six classes of events that we see on an event by event basis, we could construct cross correlation matrices and try to develop a model describing state interactions. This goes back to what Dick Horn referred to. I think it is more useful to perform experiments that test various physical hypotheses.

An interesting point can be deduced from the paper by Alain Trautmann on rat muscle. He looked at the substate in the presence of curare, which acts as both an agonist and as a channel blocker. Apparently, the subconductance state is not blocked by channel blockers. The main state durations are reduced because the channel is being blocked. If one assumes a similar blocking rate for the subconductance state, one would expect its duration to be reduced also. This was not so. Because subconductance states appear to be present in channel block experiments (see our Fig. 3), we suggest that the subconductance state is not blocked by channel blockers. This tends to contradict the hypothesis put forth in our paper, that a subconductance state is simply a drop in a well in the ion permeation energy profile.

TAKEDA: Alain Trautmann and I have examined further the agonist effects of curare in rat myotubes. The full size events show open channel block. In contrast, as you suggest, the substates are not channel blocked, as their average lifetime is not a function of curare concentration. We also observed that the frequency of events, including substates, increased from ~5%–40% as the curare concentration was raised from 10 to 100 μ M. In your preparation, is the occurrence of substates concentration dependent?

AUERBACH: We have not looked at that at all.

TAKEDA: Trautmann and I also showed that when the concentration of ACh was varied between 50 and 200 nM, there was a greater excess of brief events at the lower agonist concentration. This is as expected from the Colquhoun and Sakmann (1981) model.

AUERBACH: For SubCh and ACh, the lifetimes of the substates and the burst vary in a similar way, with the lifetimes in SubCh about twice as long as in ACh. However, over a series of agonists the relationship between length of burst and the length of substate as a function of agonist does not seem to hold. There are agonists which have short lived bursts but long lived substates.

FISHMAN: There is very little discussion about the artifacts that might arise from the patch isolation process. If there is fixed charge in the glass there should be bulk flow due to electro-osmotic effects. If this flow does not occur, then the pressure at the patch should vary as a function of voltage. With increasing complexity in patch current records, we need to have an explanation of how these incredible seals are formed, as well as a critical examination of the phenomena that can occur in the seal. Without such knowledge, we cannot proceed with confidence in analyzing data which become more complex with each additional experiment.

AUERBACH: Yes. There are artifacts associated with the patch clamp, namely, in sodium channels there are shifts in the voltage dependence of the activation-inactivation kinetics due to excision. Also, fixed charges on the glass may relate to the gradual disappearance of channels from the patch. Channels might diffuse and stick to the glass, which would cause them to lose their activity. However, since subconductance states are present in reconstituted nicotinic channels, I think they are not an artifact of the patch clamp.

EISENMAN: It is possible that the loss of channels from the patch is caused by the channels binding to the glass. However, glass is soluble, and therefore the tips of your pipettes are probably becoming saturated with silicate. A control experiment might be useful to determine if sodium silicate acts as an interfering species.

JAKOBSSON: In our Brownian dynamic simulations we see that a channel is empty for a significant fraction of the time. Thus, a possible explanation of these ultrafast spikes towards zero conductance might be a channel that was open but happened to be temporarily empty of charge carriers.

AUERBACH: For 50 μ s?

JAKOBSSON: Well, it would be a fairly infrequent event. What is the frequency of occurrence of the ultrafast spikes?

AUERBACH: That is quite variable. Sometimes we see very few, and sometimes five or six per burst. If your explanation were the case you would expect the spike frequency to be rather invariant.

JAKOBSSON: A possible explanation for the excess variance seen in the noisy zero conductance state would be the movement of ions trapped inside a closed channel. In contrast, a noise-free zero conductance state would mean the channel is closed and empty of ions.

AUERBACH: That is possible. Our simple, two-state analysis of the excess variance of the zero conductance state indicates that the excess variance could be due to unresolved, very rapid openings that did not cross our half amplitude detection criterion. In contrast, the subconductance state variance could not be modeled by a two-state model (gaps within gaps) so something more complicated seems to be going on here.

KOLB: We have been working three years with the same system, embryonic chick muscle cell, and we have not seen this type of substate up to now. Is the frequency of occurrence of these substates a function of the ACh concentration?

AUERBACH: We have used concentrations of ACh from 50 to 250 nM, but we have not done any kind of systematic analysis of the variation in burst parameters with concentration. As for not seeing the substate, you have to look pretty hard; it is fairly short lived, and it is very small. If you have a 3 pA channel, the subconductance state is $\sim 10\%$ of this, or only 300 fA (femtoamps). Also, the substate amplitude is variable. In cases where subconductance gaps are not apparent, it is not clear whether the substate gaps are faster and therefore bandwidth limited, or whether the probability of entering the substate state is small, or whether the conductance of that substate is closer to zero.

KOLB: Is the excess variance of the ground state or of the substate a function of ACh concentration?

AUERBACH: We haven't looked at this.

RAE: I have been recording from apical membrane channels in enzymatically untreated, mature lens epithelium. I have seen 13 channels, and at least half of them show subconductance levels, so subconductance levels may occur more often than presently thought. Some of these channels have large conductances, and allow recording bandwidths of up to 30 kHz. Even at this bandwidth in a single record, the shape of the trace when the channel is open may range from flat to ramps to dips. Rick Levis has modeled these channels using linear kinetic schemes that included

flickering processes well beyond the resolution of the clamp circuit. His schemes could successfully predict the unusual channel shapes seen. Therefore, it seems questionable to argue about the number of states present when there are probably additional states hidden by present bandwidth limitations.

AUERBACH: The corner frequency of the substate noise power spectrum is lower than 750 Hz, which is as low as we could go without losing measurable events. So, there are slow interconversions taking place. The corner frequency of the zero current gap noise is above 3 kHz, which is as fast as we could reliably measure. Thus, the noise of zero current gaps seems to be dominated by fast events, and the excess variance of main and substate events seems to be dominated by slower processes.

MAKOWSKI: Why is the noise level higher for a conducting channel than for a nonconducting channel? Why do you think there should be noise in a nonconducting channel?

AUERBACH: There are two unavoidable sources of "excess" open-channel noise: the thermal noise produced by the increase in channel conductance, and the "shot" noise produced by the current flow. The excess noise in the fully open and subconducting states of the nicotinic channel, however, cannot be accounted for by either of these sources.

First, the variance of the channel noise is greater than the sum of the expected thermal and shot noise variances. Second, the cutoff frequency of the predominant component of the channel noise is in the audio range, while thermal and shot noise are white. The conclusion, then, is that much of the channel noise arises from a channel repeatedly switching between conformational states having different conductances.

Our analysis of the subconductance states' noise indicates that simple band-limited subconducting-to-nonconducting transitions cannot account for the excess variance. In contrast, our analysis suggests that band-limited conducting-to-nonconducting transitions could account for the noise during zero current gaps. If so, the true mean current of these gaps must not be zero.

MAKOWSKI: What is the level of instrumental noise? What is the level of noise you see in your experiment? Is it significantly different?

AUERBACH: We use a 10-G Ω feedback resistor, so our noise is equivalent to a 5-G Ω feedback resistor, or something under 10^{-29} A²s. Depending on the bandwidth that we use, in our best records our peak-to-peak noise is about one third of the channel height, so it is ~ 1 pA peak-to-peak at the higher bandwidth recordings.

I am just guessing here, but the fact that the substate duration is not voltage dependent may mean that the site on the protein responsible for the subconductance is outside of the potential field. Because substate lifetime is agonist-dependent, the subconductance site may be near the agonist-binding site or near whatever the linkage is that controls the gating phenomenon. Finally, the apparent inability of channel blockers to block subconductance states indicates that the ion-binding site is not the same as the subconductance site. These are hypotheses that need to be tested.

MAKOWSKI: To work on the structure of the channel in the open state, the closed state, and perhaps some intermediate states, we have to be able to isolate the structures in each state in stable form. Do you have any thoughts about blocking the ACh receptor stably in intermediate states?

AUERBACH: Jorge Hidalgo, who is a graduate student in Fred Sachs' lab, has tried applying glutaraldehyde to Ca-activated channels in the hope that it would cause them to latch up in one state or another. The results were that over a two-day period no such latching occurred.

YEANDLE: Are these subconductance states due to channels forming between the membrane and glass seal? Do you ever see quiet patches that appear not to contain protein channels?

AUERBACH: I have seen quiet patches when I have hyperpolarized the membrane without ACh in the pipettes. You see these channels in reconstituted systems without a patch clamp. You see conductance states, glitches, and complicated behavior.

ANDERSEN: It seems that it is not meaningful to speak of the "conductance" of the substates or of the open state. You imply that what we can resolve as "states" with present electronic limitations are in fact groups of states with similar conductances. For example, "the" open state might consist of three open states that interconvert too rapidly to be resolved as separate states, but can be seen as excess variance. Therefore, one should give the mean conductance as well as the frequency characteristics of each state.