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Multichannel Recordings from Membranes Which Contain Gap Junctions. II. Substates and Conductance Shifts

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ABSTRACT Substates which can last up to several seconds are found in the 100-pS channel of the earthworm septum, a putative gap junction channel. The conductance of these substates is highly variable from preparation to preparation, and they are found at almost every fraction of the whole channel conductance. Another phenomenon seen in multichannel recordings is the "conductance shift": here the current passed by several open channels differs from an integral multiple of the current when only one channel is open. These shifts can be modelled by 1) a resistance in series with the channel or 2) long-lived substates. Each of these models fails in particular cases to explain either the magnitude or direction of the shifts. It is possible that both effects are simultaneously present.

INTRODUCTION

The introduction of patch techniques has enabled observation of the properties of single channels. Many patches contain a number of these channels; data from such multichannel patches form a convenient intermediary between 1) observations of a channel protein in isolation under the patch clamp (the microscopic record) and 2) recordings of the behavior of an ensemble of such channels under whole cell clamp (the macroscopic record).

In this paper, we study multichannel patches excised from the septum of the giant fibers of earthworm. The septal membranes patched contain gap junction channels in small clusters (Kensler et. al., 1979; Brink and Dewey, 1978). It is difficult, for anatomical reasons (Manivannan et al., 1992), to get many one-channel patches in this preparation. Thus it is necessary to analyze multichannel recordings as well to get information on both the kinetic or gating properties and the conductance of the gap junction channel. Manivannan et al. (1992) have shown the utility of such multichannel patches in finding the coexistence of "similar" channels, i.e., channels with the same selectivity but differing open probabilities. This analysis also indicated that cooperative behavior, i. e., interaction between these putative gap junction channels may occur in these multichannel patches. The above observations were made on the basis of analysis of the amplitude histograms of all the digitized points in the current trace. In this paper we analyze some of the conductance data from such multichannel recordings.

The conductance of a single channel is generally thought of as being quantized; thus if the channel is shut, its conductance is zero, while, if it is open, its conductance is a fixed quantity. An important exception to this statement derives from the observation that so-called "substates" are seen in recordings from every channel type (see e.g., Patlak (1988) for references). These substates are seen in short time periods where the apparent conductance is a fraction of the "fully open" or quantal conductance. Explanations of this behavior vary from fast blocking to changes in channel conformation (Läuger, 1983). For the 100-pS channel, we find that substates are evident in multichannel records with few channels as well as in the rare single channel recordings.

In the absence of substates, the composite conductance of a patch consisting of a number of identical or "similar" channels should be an integral multiple of the single channel quantal conductance. Analysis of amplitude histograms from the earthworm preparation indeed shows that the currents through many simultaneously open channels are not integrally related to the current through a single open channel. We refer to this phenomenon as a "conductance shift." We present data in which we observe shifts both in cases when the channels in the patch have identical probabilities and when they are only "similar," i.e., have different open probabilities. In what follows, we assess two possible explanations for the "shift" phenomenon: 1) a resistance in series with the channel and 2) high-probability substates.

MATERIALS AND METHODS

The methods used to directly record multichannel data from membranes containing gap junctions are those used in Brink and Fan (1989). The procedure used for recording and processing data are those presented in Manivannan et al. (1992). Several different solutions are used in the recordings. Solution X contains 135 mM CsCl, 30 tetraethylammonium chloride (TEACl), 0.6 mM EGTA, 0.1 mM $CaCl_2$, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 1 mM $CoCl_2$, and 1 mM $NiCl_2$. In some experiments 2 mM $ZnCl_2$ was also used. Solution Y is the same as solution X, except that 170 mM CsCl is used instead of 135 mM CsCl. Solution 2Y contains 340 mM CsCl but is otherwise identical to solution Y. In what follows, we use mnemonics such as X/2Y, for example, to refer to the pipette/bath solutions, when the pipette contains solution X and the bath has solution 2Y.

RESULTS

Experimental

Long-lived substates

The gap junction channel from the earthworm septum exhibits a variety of substates which can sometimes last from

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several hundred milliseconds to seconds. As noted in the introduction, single-channel records are an exception in this preparation; we have been able to obtain only two patches with only one channel. On the other hand, we have over 25 multichannel recordings from this preparation with recording times ranging from 5 to 30 min. As illustrations of substates, we show the two one-channel records as well as a single two-channel record.

Fig. 1 *a* shows an amplitude histogram of a 2-min segment of a X/X single-channel record from a patch (031489) at a holding potential of 70 mV. The data is filtered at 500 Hz and sampled at 180 μs. The histogram shows three conducting peaks in addition to the peak corresponding to zero current. These conducting peaks are at 2.5, 3.66, and 4.84 pA. There are a total of $3 \times 2 = 6$ possible transitions from any peak to any other peak. If these peaks were caused by three distinct channels, then three of the six transitions will occur not at all, or very rarely, as a result of superposition of transitions. A segment of the trace is displayed in Fig. 1, *b* and *c*; the dashed lines are at currents corresponding to the peaks in the histogram, namely at 0, 2.5, 3.66, and 4.84 pA. Some transitions are indicated in Fig. 1, *b* and *c*, by the arrowed vertical lines. In Fig. 1 *b*, these labeled transitions (from left to right) are from 3.66↔0 pA, 2.5→3.66 pA, and 4.84↔3.66 pA. Fig. 1 *c* shows the labeled transitions (again from left to right) 3.66↔2.5 pA, 2.5→4.84 pA, 4.84↔0 pA, and 2.5↔0 pA. This evidence for all six transitions implies that the conducting states at 2.5, 3.66, and 4.84 pA are conducting states of the same single channel. The substate conductances are at 52 and 76% of the maximal conductance of 69 pS (4.84 pA/70 mV). In Fig. 1 *c*, the presence of an additional brief substate at 0.7 pA can also be seen; this substate occurs rarely, and indeed there is no peak in the histogram around 0.7 pA. The probabilities of occupancy of the 0, 2.5, 3.66, and 4.84 pA states are 0.047, 0.37, 0.36, and 0.22, respectively, and the mean times spent at each state are 16.3, 19.2, 12.2, and 15.5 ms, respectively, By fitting the histogram to a sum of Gaussians (fit not shown), we can estimate the thermal noise and the extra noise contributed by the open channel in the various conducting states. The background thermal noise has a standard deviation (S.D.) of 0.2 pA. The additional open noise has a S.D. of 0.15 pA; this S.D. is the same for all the conducting states. The theoretical equilibrium Nyquist noise and the shot noise are comparable, both with a S.D. of ~0.03 pA, which is only one-fifth of the observed open noise. Sigworth (1985) has noted a number of possible causes for large open noise in AchR channels, including closely clustered substates and conformational fluctuations. We interpret the near equality of the S.D. of the open noise in all the conducting states in Fig. 1 as evidence of fast background conformational transitions, which take place regardless of the conductance of the pore.

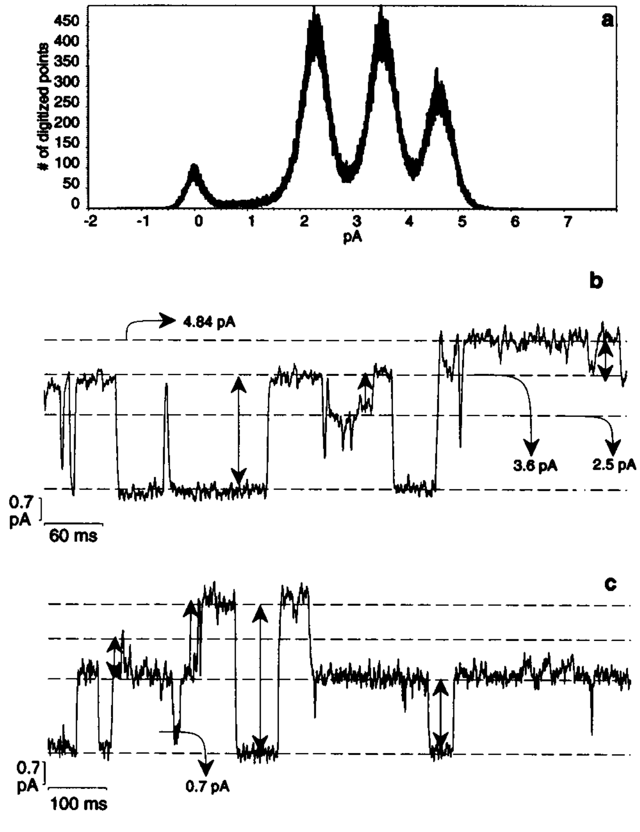

FIGURE 1 (*a*) Amplitude histogram of 2 min of a single channel X/X record held at 70 mV. The peaks correspond to long-lived conducting states at 2.5, 3.66, and 4.84 pA. (*b* and *c*) Transitions from every peak to every other peak; the presence of all six possible transitions demonstrates that all three peaks correspond to substates.

The top panel of Fig. 2 is the trace of 4 min of a recording from a X/X patch (101988) at a holding potential of 60 mV. This patch starts with three channels; the number drops down to two active channels. In the 4-min shown, however, the two channels are open simultaneously for only 130 ms (0.06% of the time). As the second channel is closed most of the time, the trace can be effectively regarded as a one-channel record. Fig. 2, *a*–*d*, are blow-ups of the regions similarly marked in the top panel. The dotted lines in all the panels are at 0 pA and at 7 pA. In all panels, we see evidence of a number of conducting states apart from the dominant one at 7 pA. As we do not, in this record, find evidence for all possible transitions between all these conducting states, we have adopted a different strategy to show that there are substates. In the beginning of the trace in Fig. 2 *d*, note that there is a transition from the nonconducting state (0 pA, the *lower dotted line*) to the conducting state at 7 pA. If we rule out the possibility that two channels will open simultaneously, then this transition shows that 7 pA is a conducting state for a single channel. Now any state with *smaller* conductance reached from a conducting state where only one channel was open must be a substate of the same channel. Following the trace in Fig. 2 *d*, we see, by this argument, that the trace steps briefly down into a subconducting state at 5 pA before a prolonged sojourn at a subconductance at 3.8 pA. Inspection of the data shown in Fig. 2, *a*–*c*, by this method reveals numerous other substates (e.g., at 2.8 pA, Fig. 2 *b*) which are reached from the main conducting state at 7 pA. The fact that there are few or no transitions between most of these substates and the nonconducting state may be related to some

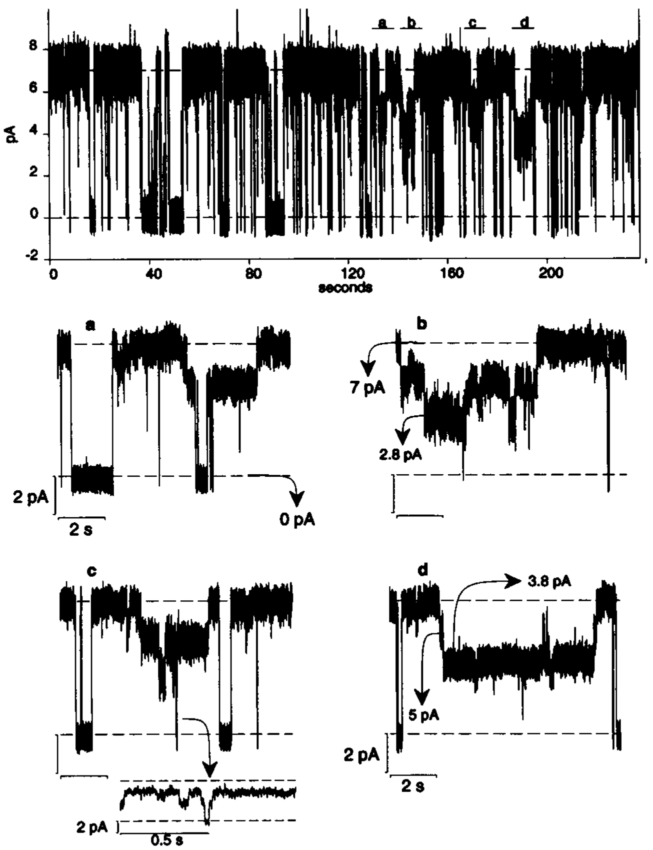

FIGURE 2 The top panel is the trace of 250 s of a X/X patch where two channels are present. The holding potential is 70 mV. As the two channels are open simultaneously only for 0.06% of the time (130 ms), we regard this as a single-channel record. (*a–d*) Enlargements of the regions correspondingly marked in the top panel. Transitions from the dominant conducting state at 7.0 pA downward to substates can be seen. There are a large number of substates, including some that are close to 7 pA. (*b* and *d*) Evidence for extended sojourns (1 s or longer) in the substates.

irreversible behavior in this channel (see also Sachs (1983)). We note from the trace (Fig. 2, *a* and *d*) that there are extended stays (of the order of 1 s) in some of the substates; as an example note the duration of the substate at ~3.8 pA in Fig. 2 *d*. This substate persists for over 1 s. This represents what we call a long-lived substate.

Fig. 3 *a* is an amplitude histogram of 4 min of a multichannel record (033189) of an X/X patch at a holding potential of 87 mV; a small portion of the trace from this patch can be seen in Fig. 1 of Manivannan et al. (1992). The dashed line in Fig. 3 *a* is a fit made by assuming two identical channels. Each channel in the fit is assumed to have three conducting states at 54, 72, and 87 pS with probability of 0.015, 0.115, and 0.255, respectively. The background noise S.D. is 0.3 pA, while the three conducting states add extra noise with S.D. of 0.35, 0.52, and 0.43 pA, respectively. The three panels labeled *b*, *c*, and *d* offer evidence that there are indeed substates by showing step-down transitions (*vertical arrows*) to the smaller conductance states (shown by *dotted lines*) following a bigger step-up transition to the largest conducting state (87 pS).

In Fig. 3 *a* the open noise S.D. varies from substate to substate (compare discussion of Fig. 1, above). There are two possible interpretations: 1) the transport mechanism for ions varies in the different substates, which makes the noise S.D. vary as well 2) the substates used in the fit are themselves composites, i.e., each apparent substate consists of several closely bunched substates that cannot be distinguished due to thermal and shot noise. It may be possible to distinguish between these possibilities through the power spectrum of the noise.

If each channel had three conducting states, then, with two channels open, we would generally expect to see at least six different conducting states. Some of these substates with two channels open can be seen in Fig. 3 *e*. The low signal-to-noise ratio when two channels are open precludes clear identification of distinct substates; we only note that there are at least seven visible in Fig. 3 *e*.

Analysis of records from 15 multichannel patches for a total of 42 holding potentials shows that the number of substates, their conductance, and the probability of their occurrence vary quite drastically from record to record. At least two substates are seen when only one channel is open in every record examined, and ~90% of the records have three

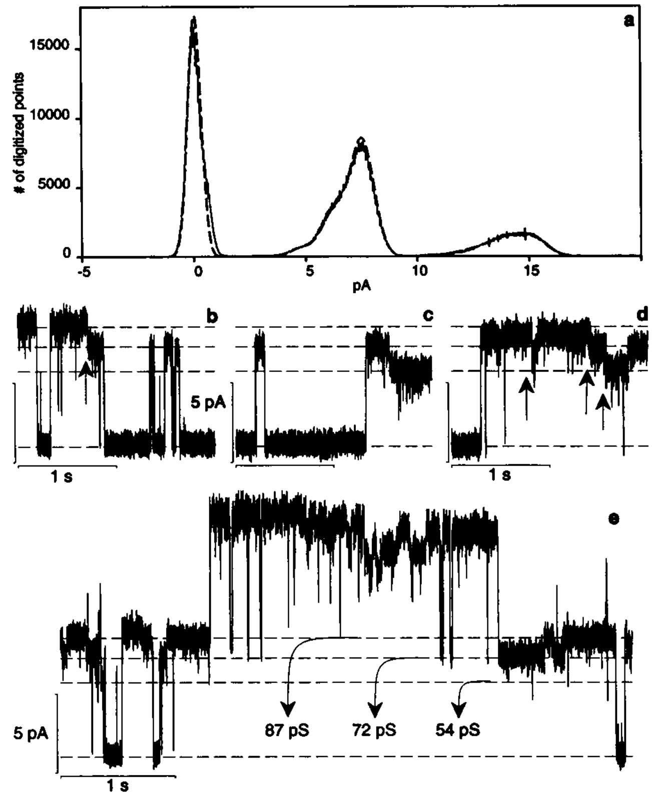

FIGURE 3 (*a*) An amplitude histogram of 4 min of a X/X patch at a holding potential of 60 mV. The dashed line in this panel is a fit made by assuming two identical channels, each with three conducting substates (see text for fit parameters). As in Fig. 2, transitions between the main conducting state at 87 pS and the substates at 54 and 72 pS (in *dotted lines*) can be seen in *b*, *c*, and *d*. (*e*) Presence of large number of substates when two channels are simultaneously open.

or more substates. In the multichannel histograms shown in Figs. 4–6, the substates cluster close to the main conducting peak; this results in a large apparent extra noise added by the channel when it is open. When focusing on aspects of the data other than substates, as in the section to follow in this paper, we have picked records where the substates do not appear as independent peaks in the histograms; such records constitute a majority of the recordings. Even in such cases, the presence of the substates can sometimes be seen in the skewing of the peaks in amplitude histograms (see, e.g., Fig. 2 in Manivannan et al. (1992)). In many of the other recordings, the high probability of the substates, as in Fig. 3, make inadequate any fits that assumed that each channel had only closed and open states. Further, as can be seen from Figs. 1, 2, and 3, the ratio of substate conductance to the dominant conductance varies from patch to patch. The only consistent feature across all records is that the substates have an average lifetime from ~10 to ~50 ms (Fig. 1, *b* and *c*, and Fig. 3 *c*, insert), a time that is comparable to the lifetime of the unitary conductance. It is in this sense that we refer to the substates as "long-lived." Lack of knowledge of the control variables that influence the characteristics and appearance of the substates precludes a more quantitative assessment of this phenomenon at the present time.

Conductance shifts

As noted in the Introduction, we have observed that the current passed by several open channels is not an integer multiple of the current through a single open channel. These "conductance shifts" can be readily observed in the amplitude histograms of the current through the patch. The most prevalent shift is negative, i.e., the phenomenon of *n* simultaneously open channels passing a current which is less than *n* times the current through a single open channel. The second possibility is a positive shift, where the current through a newly open channel is more than the current through an already open channel; this was not observed with regularity. The magnitude of the positive shifts was also always less when compared to those of the negative shifts. Cases where no apparent shift could be observed were rare.

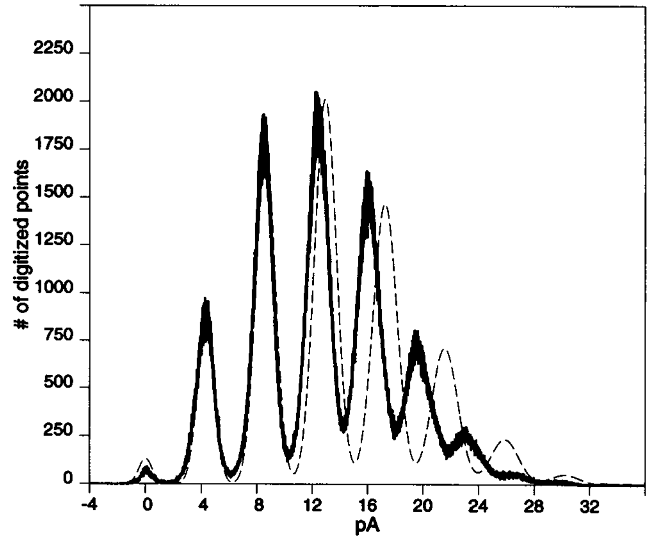

FIGURE 4 The conductance of *n* simultaneously open channels is not an integer multiple of the conductance of one open channel. The holding potential is 45 mV; the duration of the X/X record is 7 min. This figure shows the fit in dotted lines to an amplitude histogram when all the channels pass the current associated with the first peak. This demonstrates that as the number of open channels increases, the current is less than that which would be expected if all channels passed the same current as a single open channel. One channel with an open probability of 0.81 and 9 channels with an open probability of 0.27 are used for the fit; these numbers are obtained from a maximum likelihood algorithm.

Negative shifts with similar channels

Fig. 4 shows the amplitude histogram of a record from a X/X patch (033089) at a holding potential of 45 mV. (see also Manivannan et al. (1992)). The dotted line shows a fit when the unitary current for a single channel is taken to be the peak of the Gaussian (= 4.12 pA) in the histogram corresponding to one channel open. The number of channels actually observed during the 7 min of recording was 9; the fit was produced by assuming one channel with open probability = 0.81 and 9 channels with open probability = 0.27. The fits here and in other figures were optimized for maximum likelihood using the algorithms of Ramanan et al. (1992); this algorithm allows for two types of channels. Note the increasing displacement in the position of the peaks of the fit from the data as the number of open channels increases. The simplest way to explain this shift is to postulate that the channel with the probability of 0.81 has a larger conductance than the other 9 channels with probability of 0.27. We discuss this possibility further in Theory below.

Positive shifts with similar channels

Fig. 5 is an amplitude histogram from a Y/Y patch (072490) where two channels are seen to open during the 2 min of recording. The holding potential was 60 mV. The fit assumes that there is one channel with open probability of 0.98 and one channel with open probability of 0.60. The current when both channels are open is assumed to be two times the current when only one channel is open. Note that the peak in the histogram corresponding to two channels simultaneously open occurs to the right of the corresponding peak in the fit, i.e., the actual current is greater than the expected current.

Negative shifts with identical channels

Fig. 6 shows negative shifts with identical channels. A maximum of three channels were seen simultaneously open during the 3 min of recording from a Y/2Y patch (072590) at a holding potential of 45 mV. The fit (in *dotted lines*) is made by assuming that all three channels have an open probability of 0.375. As in Fig. 4, the expected currents shown in the fit are greater than the actual currents when more than one channel is conducting.

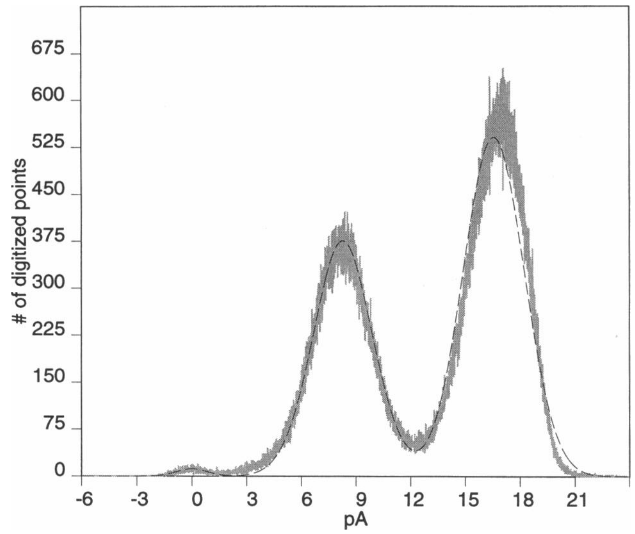

FIGURE 5 This figure is an amplitude histogram of 2 min of a two-channel Y/Y record. The dotted lines represent the fit when both channels pass the same current as a single open channel. Note that the actual peak in the amplitude histogram when both channels are open is to the right of, i.e., greater than, the expected peak shown in the fit. The fit was made with one channel of open probability of 0.98 and one of open probability of 0.60.

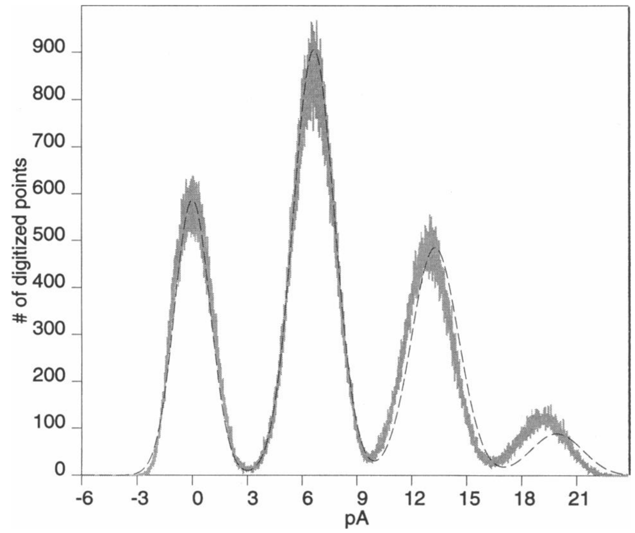

FIGURE 6 This figure demonstrates the nonunitary multiple channel conductances when all the channels have identical open probabilities. The amplitude histogram of 3 min of a Y/2Y multichannel record is fitted by assuming three channels with an open probability of 0.375 and all channels are assumed to pass the same current as a single open channel. Note that the actual currents when several channels are simultaneously open are lesser than the expected values.

As a check against instrument artifacts, we examined multichannel recordings from excised patches from avian plasma cells from the same set-up as, and contemporary with, the recordings considered here. The channels in these patches were maxi-K channels (Brink et al., 1990). We examined data from five patches at various holding potentials for a total of 10 recordings, as summarized in Table 1. Small negative conductance shifts were also seen in these recordings. The last column represents the equivalent resistance in the pipette that would cause these shifts (see Theory below). The shifts were generally three to 10 times smaller than those observed in recordings of nexal membranes.

Table 1 Conductance shifts seen in multichannel maxi-K preparations

Patch	No. of voltages	No. of channels	Equivalent series resistance
			MΩ
051090B	2	6	50
032889A	3	3	30–50*
012389A	1	2	50
012389B	3	5	20–50*
012389C	1	2	20

* Variation from different holding potentials

Theory

Series resistance

The simplest cause for the behavior seen in Fig. 4 is a constant resistance in series with the channel. If the channel in the record is a gap junction channel, then the membrane in the patch is a bimembrane; the single membrane not adhering to the pipette tip can therefore flap to produce a constriction which causes a series resistance in the circuit. Let g_n be the apparent conductance when n channels are simultaneously open, given by

$$\frac{1}{g_n} = \frac{1}{nG} + \frac{1}{G_s}, \tag{1}$$

where G is the actual conductance of an open channel and $R_s = 1/G_s$ is the resistance in series. The nth conductance shift Δg_n corresponding to the $(n + 1)$th peak is defined as

$$\Delta g_n \equiv n g_1 - g_n. \tag{2}$$

Equation 1 can now be rearranged to yield

$$\frac{\Delta g_n}{g_n} = (n - 1)\frac{G}{G + G_s}. \tag{3}$$

Hence, a plot of $(\Delta g_n/g_n)$ vs. $(n - 1)$ should be fit by a straight line from whose slope the series resistance can be found. Fig. 7 shows such a plot for several records from a single patch (033089) at different holding potentials V_H. The equivalent series resistance for this patch, as calculated from the slopes of the best linear fits, ranges from a minimum of 0.14 GΩ at 45 mV to a maximum of 0.88 GΩ at 90 mV, assuming a channel resistance of 10 GΩ. It can be seen that there is a systematic increase in R_s as the holding potential is increased.

If the series resistance proposed above is to serve as a satisfactory explanation for the shift phenomenon seen in multichannel records, then the current observed when m channels are open must be less then m times the current when only one channel is open. To produce a positive shift such as that seen in Fig. 5, the series resistance would have to be

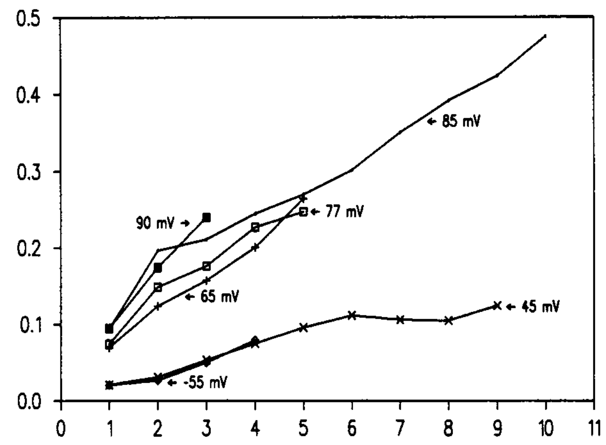

FIGURE 7 Plot of the relative reduction $\Delta g_n/g_n$ in the current per channel against $(n - 1)$, where n is the number of open channels. If the reduction in current was due to a resistance in series with the patch, then this should be a linear relationship. All the data shown is from a single patch at various potentials. Using Eq. 3, we can extract the ratio of the series resistance to the channel resistance from the slope. This ratio increases from ~0.01 at 45 mV to ~0.08 at 90 mV. For a channel resistance of 10 GΩ, this is a series resistance of 100–800 MΩ.

negative; such unphysical negative resistances indicate the presence of some phenomenon other than the access resistance proposed above. We note that the magnitude of the small negative shifts observed in multichannel patches of the Maxi-K channel (see Table 1) made in the same experimental setup is readily explainable in terms of a small pipette resistance (≤50 MΩ).

Long-lived substates

Manivannan et al. (1992) have shown that there can be "similar" channels in a patch. Such similar channels have identical selectivity and conductance, but possess different open probabilities under apparently identical external conditions. We now demonstrate one scenario where the apparent measured conductance G of a single channel depends on its open probability P.

Consider a channel with two conducting and long-lived states. Assume that the presence of the individual conducting states is masked due to Gaussian noise and that only their contribution to the effective open current is detectable. Fig. 8, *a–c*, shows three different cases where the ratio of the occupancy of the two conducting states varies with the total open probability P of the channel. The long vertical dashed lines show the shifting of the perceived open current as the relative occupancy of the two states changes. Such a shift would manifest itself as a dependence of the effective conductance G on the open probability P.

We can readily postulate other scenarios where G is a function of P. Whatever the reason, such a dependence of the effective open conductance G on the open probability P can be quantified by performing a number of experiments on patches containing only one channel, and plotting G against P. Such a dependence would also cause conductance shifts in a multichannel record, as shown below.

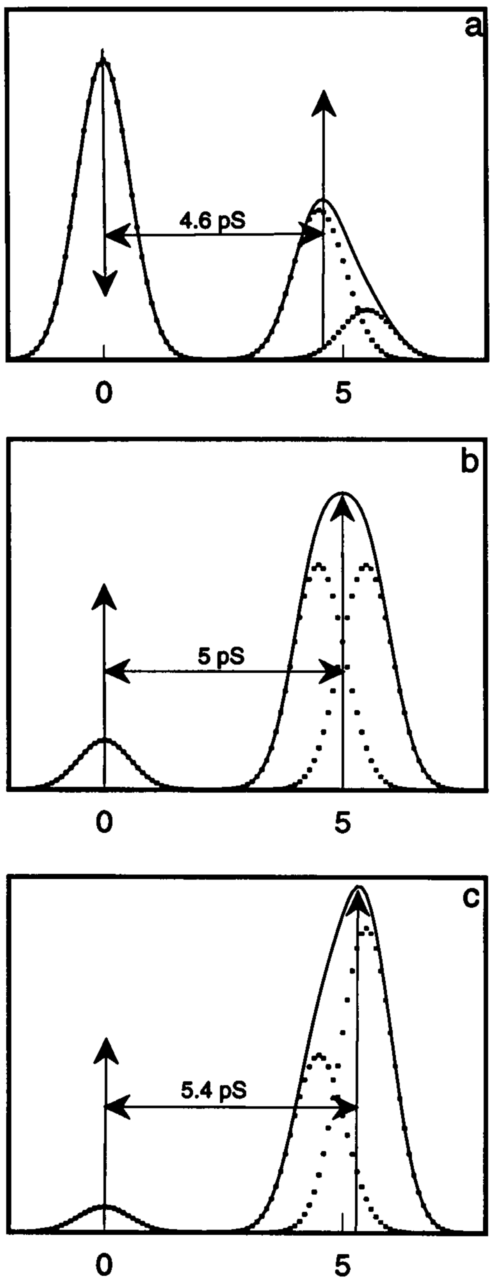

FIGURE 8 This figure illustrates the influence of substate occupation probabilities on the perceived conductance of the channel. There are two substates, one (A) with a conductance of 4.5 pS and the other (B) with a conductance of 5.5 pS. Both substates have a Gaussian S.D. of 0.6 pS. In *a*, substate A has a probability of 0.3 and substate B has a probability of 0.1. The effective peak is at 4.6 pS. In *b*, both substates have a probability of 0.45; the effective current is the mean of the two conductances at 5 pS. In *c*, the probabilities are 0.35 and 0.6 for substate types A and B, respectively, the net effect is a conductance of 5.4 pS.

The dependence of effective single channel conductance G is represented as below in the probability density function of the conductance of a single channel with only one conducting state:

$$f(\mathscr{g}) = (1 - P)\delta(\mathscr{g}) + P\delta(\mathscr{g} - G), \qquad (4)$$

where the effective open channel conductance G is a function $G(P)$ of the open probability P. Our main result is the following.

THEOREM: *Consider an assortment of N channels, all described by Eq. 4 and having open probabilities* P_k, $k = 1, \ldots, N$. *As in the previous section,* g_k *denotes the mean conductance when k channels are open. If the single channel conductance* $G(P)$ *decreases monotonically with open probability P, then the kth conductance shift*

$$\Delta g_k = k g_1 - g_k \leq 0 \tag{5}$$

with equality holding when all the open probabilities are equal. The reverse inequality can be proved for monotonic increase of $G(P)$ *with P.* (A proof of this assertion may be found in the Appendix.)

A motivation for the result can be provided as below. The largest contribution to the conductance when only one channel is open is provided by that channel with the highest open probability. The conductance when two channels are open is mainly determined by the two channels with the two highest open probabilities. If the conductance of the channels increases monotonically with increasing open probability, then the result above appears to be a plausible one.

Consider a hypothetical application to gap junctions. These channels are distinguished by the fact that they are formed by two hemichannels at the apposition of two membranes. Assume that each hemichannel is individually gated, so that both have to be open for the channel to conduct. Further, let there be a leak at the junction between the hemichannels into the interstitial gap. The patch clamp situation can then be depicted as below:

$$f(\mathscr{g}) = q_1 \delta[\mathscr{g}] + p_1 q_2 \delta\left[\mathscr{g} - \frac{1}{R(1+m)}\right] \tag{6}$$

|------CLOSED------| |--

$$+ p_1 p_2 \delta\left[\mathscr{g} - \frac{1+m}{R(1+2m)}\right].$$

--OPEN----|

Here R is the resistance of the hemichannel, R_{leak} is the leak resistance from the center of the channel into the extracellular gap, $m = R_{\text{leak}}/R$, p_1 and p_2 are the open probabilities of the top and bottom hemichannels, respectively, and $q_{1,2} = 1 - p_{1,2}$. We normalize R to 1. Note that the open probability P of the whole channel is given by $P = p_1 p_2$. We need to put Eq. 6 into the form in Eq. 4. Lumping the closed substates together, the effective closed conductance is given by $[0.q_1 + p_1 q_2/(1 + m)]/(q_1 + p_1 q_2)$. We subtract this quantity from the open conductance $(1 + m)/(1 + 2m)$ to find the equivalent of Eq. 4, giving

$$G = \frac{1}{(1+m)(1+2m)}\left[m^2 + (1+2m)\frac{1-p_1}{1-P}\right], \tag{7}$$

where G is the mean conductance of a single channel with open probability P. We consider three situations:

1) The hemichannels are identical, implying that $p_1 = \sqrt{P}$. We find

$$\frac{dG}{dP} = -\frac{1}{(m+1)}\frac{1}{2\sqrt{P}(1+\sqrt{P})^2} < 0.$$

By the assertion above, the shifts are all negative.

2) p_1 is a constant. This implies that variation in P are caused only by variations in the probability of the bottom hemichannel. Then

$$\frac{dG}{dP} = \frac{1-p_1}{1+m}\frac{1}{(1-P)^2} > 0.$$

The shifts are now positive.

3) The bottom hemichannel is permanently open, i.e., $p_2 = 1$. This implies $p_1 = P$, thus $dG/dP = 0$, and there is no shift.

We end this section by noting that two criteria, namely, (a) the presence of long-lived substates and (b) the existence of channels with different probabilities in the same patch, must be satisfied if the above theory is to explain the observed shifts. Both are met in the channel under study. Thus the model can be treated as being plausible.

CONCLUSION

The most striking characteristic of the substates in the 100-pS channel is their duration. Inspection of the records shows that these can last from less than a millisecond to several hundred milliseconds, and sometimes for several seconds. They could thus correspond to long-lived stable conformational states of the channel macromolecule. The probability density functions (pdfs) of sojourns in open and closed states in the few single channel records show that exponentials with time constants of up to seconds are needed to fit the data adequately. In Fig. 9, we show the pdfs of open and closed states from 50 min of the recording, part of whose trace is shown in Fig. 2. Both open and closed pdfs require an exponential with time constant of ~1 s for a satisfactory fit. The substate data is thus consistent with these observations in demonstrating the existence of long-lived conformational states in the 100-pS channel protein. The same phenomenon may also be invoked to explain the presence of "similar" channels in the preparation (Manivannan et al., 1992). Here "locking" of the channel into one of these long-lived conformational states may cause the channel to appear to have a different open probability from other channels over a comparatively long time of several minutes. We note that, in addition, leakage into the gap between two adjacent cells could also produce substates (see section on long-lived substates). While our data can neither support nor contest the notion that substates are caused by channel blockade via solute binding with channel walls, the time scale is generally thought of as being too short for long-lived substates. We note that if one were

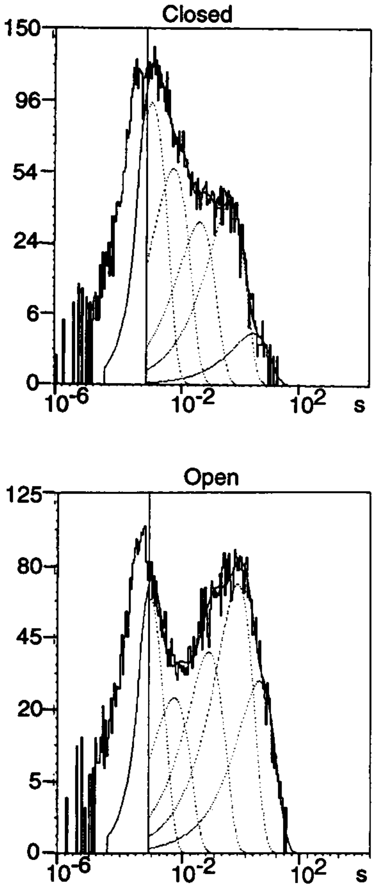

FIGURE 9 The top and bottom panels are Sigworth-Sine plots of of closed and open pdfs, respectively, of 50 min of recording of an X/X patch at a holding potential of 70 mV. This is from the same recording as that used in Fig. 2, but we have selected a portion where the contribution of the substates is minimal. The vertical line in both panels is at 720 μs, which is twice the deadtime of the apparatus (filter at 0.5 kHz). The continuous line is the fit of the sum of the exponentials, shown in dotted lines. The fit parameters for the closed pdf are: w1 = 0.421, t1 = 1.11 ms; w2 = 0.248, t2 = 5.9 ms; w3 = 0.141, t3 = 39.5 ms; w4 = 0.175, t4 = 351 ms; w5 = 0.015, t5 = 2120 ms where the w and t letters are the weights and the time constants of the exponentials summed to form the fit. Similar parameters for the open pdf are: w1 = 0.289, t1 = 0.74 ms; w2 = 0.104, t2 = 5.26 ms; w3 = 0.173, t3 = 66.6 ms; w4=.308, t4 = 555 ms; w5=.126, t5 = 2820 ms. The fits are maximized for likelihood for events longer than 720 μs by the procedure given in Sigworth and Sine (1987). The number of exponentials is increased till the change in maximum likelihood is less than 2 (Akaike's criterion). There are 7329 sojourns in open and closed states, and the probability of opening is 0.844. The mean closed and open times are 65.4 and 334 ms, respectively.

Table 2 Assessments of conductance shifts

	Channels →	Identical*		Similar		Equivalent series resistance
Row ↓	Shift →	Up	Down	Up	Down	is voltage dependent?
1	Data	N	Y	Y	Y	Y
2	Series resistance	N	Y	N	Y	N
3	Long-lived substates‡	N	N	Y	Y	Can be.
	Column →	1	2	3	4	5

* "Identical" means that the open proabilities of the channels used in the fit to the multichannel histogram are the same. "Similar" means that at least two different open probabilities were found to be necessary to fit the amplitude histogram. Y, yes; N, no.

‡ In the analysis, long-lived substates have effects that are formally identical to having channels with different probabilities and conductances in the patch.

to consider a giant composite record of the conductances, the conductance of the substates would apparently vary in a near continuum from ~40 to 100% of the unitary conductance.

We have noted that there is much variation of the properties, both kinetic and conductance, of the substates from patch to patch. The explanation of the "shifts" in terms of channels with differing substate behavior is the only one that allows for the current passed by a channel to increase when the number of already open channels increases. Due to the difficulty of quantifying the data on substates, however, this model remains untested and can at most be treated as plausible. The substate model for the shifts, however, fails to produce a shift for identical channels. The "series resistance" model does not suffer from this restriction.

The relative strengths of the various models is assessed in Table 2. Thus the data (row 1) shows down shifts with identical channels (columns 1 and 2), and also up shifts with similar channels (columns 3 and 4). If the shifts are interpreted as an equivalent series resistance, then this resistance apparently varies with the imposed voltage across the membrane (column 5). By comparison, the series resistance model (row 2, see Theory) predicts only downshifts irrespective of whether the channels are identical or only similar, and this series resistance is (by definition) voltage-independent. The shifts-by-substates model is similarly assessed in row 3. Both models suffer from a failure to explain the composite facets of the shifts found in the data; a combination of both factors may thus be responsible. We note that interaction between the fluxes through neighboring channels can also cause conductance shifts. We will assess this model in a separate paper.

Though the substates are a single-channel phenomenon, it is readily seen from Fig. 3 that they can have a substantial influence on the conductances of an aggregate of channels. The "shifts through substates" is one route where their influence may be readily observable. It is also possible that substates may be a result of protein-protein or protein-lipid-protein interaction when channels are packed together. Multichannel recordings are a natural tool for investigating these phenomena.

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APPENDIX

Consider a collection of N channels with open probabilities P_k, closed probabilities Q_k (= $1 - P_k$) and associated unitary currents G_k for $k = 1, \ldots, N$. The first peak in the amplitude histogram is associated with the closed state and is a Gaussian around zero conductance. For the second peak, the mean conductance is

$$g_1 = \frac{P_1Q_2 \ldots Q_NG_1 + \ldots + Q_1Q_2 \ldots P_NG_N}{P_1Q_2 \ldots Q_N + \ldots + Q_1Q_2 \ldots P_N}. \tag{A1}$$

Similarly,

$$g_2 = \frac{\sum\limits_{N \geq b > a \geq 1} Q_1 \ldots Q_{a-1}P_aQ_{a+1} \ldots Q_{b-1}P_bQ_{b+1} \ldots Q_N(G_a + G_b)}{\sum\limits_{N \geq b > a \geq 1} Q_1 \ldots Q_{a-1}P_aQ_{a+1} \ldots Q_{b-1}P_bQ_{b+1} \ldots Q_N}. \tag{A2}$$

Denote

$$S_r^N(A, B) = \frac{1}{r} \sum_{\substack{a_1, \ldots, a_r = 1 \\ a_1 \neq a_2 \ldots \neq a_r}}^{N} A_{a_1} \ldots A_{a_r}(B_{a_1} + \ldots + B_{a_r}), \tag{A3}$$

for collections A and B of quantities A_r and B_r, respectively, $1 \leq r \leq N$. We can derive a recursion relation for S as follows:

$$\begin{aligned} S_r^N(A, B) &= \sum_{\substack{a_1, \ldots, a_r = 1 \\ a_1 \neq a_2 \ldots \neq a_r}}^{N} A_{a_1} \ldots A_{a_r}B_{a_r} \\ &= \sum_{\substack{a_1, \ldots, a_r = 1 \\ a_1 \neq a_2 \ldots \neq a_r}}^{N} A_{a_1} \ldots A_{a_{r-1}} \left(\sum_{k=1}^{N} A_kB_k - A_{a_1}B_{a_1} - \ldots - A_{a_{r-1}}B_{a_{r-1}} \right) \\ &= \langle AB \rangle S_{r-1}^N(A, 1) - (r - 1)S_{r-1}^N(A, AB), \end{aligned} \tag{A4}$$

where $\langle G \rangle = \sum_{k=1}^{N} G_k$. Note that $S_1^N(A, B) = \langle AB \rangle$. We also define $S_0^N = 1$.

We may now write

$$g_r = r\frac{S_r^N(f, G)}{S_r^N(f, 1)}, \tag{A5}$$

where $f_a = P_a/Q_a$.

Applying the recursion relation (Eq. A4) once to both the numerator and denominator of the above equation, we get

$$g_r - rg_1 = \frac{r(r - 1)}{\langle f \rangle S_r^N(f, 1)} [\langle fG \rangle S_{r-1}^N(f, f) - \langle f \rangle S_{r-1}^N(f, fG)]. \tag{A6}$$

To prove the theorem, we need to show that the RHS of the above equation is positive when G is a monotonically decreasing function of f. As the terms outside the bracket are positive, we are concerned only with the bracketed quantity. Repeated use of the relation (Eq. A4) allows this to be rewritten as

$$\frac{1}{2} \sum_{a,b=1}^{N} f_af_b(G_b - G_a)(f_a - f_b) \times \left[\sum_{k=0}^{r-2} (-1)^{r-k} \frac{(r - 2)!}{k!} S_k^N(f, 1) \frac{f_a^{r-k-1} - f_b^{r-k-1}}{f_a - f_b} \right]. \tag{A7}$$

Define f_{ab} as the collection f excluding the quantities f_a and f_b. Consideration of the combinatorics allows us to write

$$S_r^N(f, 1) = S_r^{N-2}(f_{ab}, 1) + r(f_a + f_b)S_{r-1}^{N-2}(f_{ab}, 1) + r(r - 1)f_af_bS_{r-2}^{N-2}(f_{ab}, 1) \tag{A8}$$

It may be verified that if $S_{r-2}^{N-2}(f_{ab}, 1)$ is set equal to the term in square brackets in Eq. A7, then the relation (Eq. A8) is satisfied. We may thus write

$$g_r - rg_1 = \frac{1}{\langle f \rangle S_r^N(f, 1)} \frac{r(r - 1)}{2} \sum_{a,b=1}^{N} f_af_b(G_b - G_a)(f_a - f_b)S_{r-2}^{N-2}(f_{ab}, 1) \tag{A9}$$

As the $S_{\cdot}^{\cdot}(A, 1)$ are strictly positive quantities if the components of A are positive, the assertion is proved.

REFERENCES

Abramowitz, M., and I. Stegun. 1970. Handbook of Mathematical Functions. Dover Publications, Inc., New York.

Brink, P. R., and M. M. Dewey. 1978. Nexal membrane permeability to anions. *J. Gen. Physiol.* 69:517–536.

Brink, P. R., and S.-F. Fan. 1989. Patch clamp recordings from membranes which contain gap junction channels. *Biophys. J.* 56:579–593.

Brink, P. R., E. J. Roemer, and B. Walcott. 1990. Maxi-K channels in plasma cells. *Pflugers Arch.* 417:349–351.

Kensler, R. W., P. R. Brink, and M. M. Dewey. 1979. The septum of the lateral axon of the earthworm: a thin section and freeze fracture study. *J. Neurocytol.* 8:565–590.

Laüger, P. 1983. Conformational transitions of ionic channels. *In* Single-Channel Recording. B. Sakmann and E. Neher, editors. Plenum Press, New York. 177–189.

Manivannan, K., S. V. Ramanan, R. T. Mathias, and P. R. Brink. 1992. Multichannel recordings from membranes which contain gap junctions. *Biophys. J.* 61:216–227.

Patlak, J. B. 1988. Sodium channel subconductance levels measured with a new mean-variance analysis. *J. Gen. Physiol.* 92:413–430.

Ramanan, S. V., S. F. Fan, and P. R. Brink. 1992. Model invariant methods for extracting single-channel open and closed times from heterogeneous multichannel records. *J. NeuroSci. Methods.* 42:91–103.

Sachs, F. 1983. Is the acetylcholine receptor a unit-conductance channel? *In* Single-Channel Recording. B. Sakmann and E. Neher, editors. Plenum Press, New York. 365–376.

Sigworth, F. J., and S. M. Sine. 1987. Data transformations for improved display and fitting of fingle-channel dwell time histograms. *Biophys. J.* 52:1047–1054.

Sigworth, F. J. 1985. Noise in acetylcholine receptor currents suggests conformational fluctuations. *Biophys. J.* 47:709–720.