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Evidence that CFTR Channels Can Regulate the Open Duration of other CFTR Channels: *Cooperativity*

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Abstract. CFTR channels mediate secretion and absorption in epithelia, and cystic fibrosis is caused by their malfunction. CFTR proteins are members of the ABC transporter family and are complexly regulated by phosphorylation and nucleosides; they also influence other channel activity. Do CFTR molecules also influence one another? Cooperativity has been observed among other channels and has been suggested for CFTR. Therefore, we looked for evidence of cooperativity among CFTR channels using three independent approaches. All three methods provided evidence for cooperativity in CFTR gating. We estimated mean open times, independent of the number of channels in the patch, in multi-channel patches and showed that, on average, they increased as channel number increased. We observed many trials having larger than expected variances, consistent with cooperative gating. We also measured deviations from binomial statistics, which revealed cooperativity and further indicated that its magnitude is underestimated to an unknown extent because of masking that occurs when CFTR channel populations within a single patch have heterogeneous open probabilities. Simulations showed that the observed departures from binomial statistics were too large to have arisen by chance. The evidence that CFTR $P(o)$ increases with channel density has important functional implications.

Key Words: Cystic fibrosis — Channel gating — Calu-3 — Binomial — Open time

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[1] *Abbreviations used in this paper:* CFTR, cystic fibrosis transmembrane conductance regulator; $P(o)$, open probability; N, number of active channels in patch.

Introduction

The cystic fibrosis transmembrane conductance regulator (CFTR)[1] is a 1480 amino acid membrane protein. Mutations in CFTR can cause cystic fibrosis, a prevalent genetic disease. CFTR is a small conductance anion channel that is gated by cAMP and ATP (Anderson et al., 1991; Bear et al., 1992; Drumm et al., 1990; Rich et al., 1990). Before its function as an ion channel was confirmed, CFTR was called a 'conductance regulator' (Riordan et al., 1989) to allow for the possibility that the chloride conductance missing in CF (Quinton, 1983) was either extrinsic or intrinsic to CFTR. Although its intrinsic ion conductance is well established (e.g., Anderson et al., 1991; Bear et al., 1992), numerous studies continue to explore CFTR's ability to influence other molecules including ENaC (Stutts, Rossier & Boucher, 1997), gap junctions (Chanson, Scerri & Suter, 1999) and aquaporin 3 (Schreiber et al., 1999). For an extensive review *see* Schwiebert et al. (1999).

Because of the evidence that CFTR can regulate other channels, we wondered whether CFTR channels might also influence one another (cooperativity). Other channels have been shown to exhibit cooperativity (Iwasa et al., 1986; Manivannan et al., 1992; Neumcke & Stämpfli, 1983), and hints of cooperative behavior have been seen in CFTR gating. Kartner et al. (1991) reported slow, wave-like activity in multi-channel patches that may have been caused by interactions among CFTR channels. Haws et al. (1992) and others (Bear et al., 1992; Fischer & Machen, 1994) reported near simultaneous openings and closings of CFTR channels. However, none of this evidence has been explicitly analyzed. Cooperative interactions may be incorrectly inferred when channel behavior is correlated for other reasons, or may be underestimated if patches contain channels with unequal open probabilities (Manivannan et al., 1992).

Wang et al. (2000) recently showed that CAP70 is

expressed in the Calu-3 cell line and can mediate CFTR-CFTR interactions, leading to increased channel open time. These results suggest that CFTR channels should display cooperativity in this cell line. We investigated CFTR from Calu-3 cells in multi-channel patches to determine if CFTR channels gate cooperatively. We used three independent approaches to assess cooperativity. The first uses a technique for estimating mean open times in a multi-channel patch (Horn & Lange, 1983), independent of the number of channels in the patch. The second looks at the distribution of the variance of the trials. The third uses deviations from binomial statistics to estimate cooperativity (Manivannan et al., 1992). Results from all methods indicate that cooperativity exists in CFTR gating. The binomial analysis further indicates that the magnitude of cooperativity is underestimated to an unknown extent because of masking that occurs when CFTR channel populations within a single patch have heterogeneous open probabilities. The specific kind of cooperativity we found in this study was the prolongation of the open-duration of CFTR channels by other CFTR channels. Some of these data appeared in abstract form (Krouse, 1995).

Materials and Methods

Calu-3 cells were obtained from American Type Cell Collection and were grown in Eagle's MEM w/Earle's BSS and 10% FBS (UCSF Cell Culture Facility) at 37°C in an atmosphere of 5% CO_2 and 95% air. For patch-clamp studies, cells were plated at low density on 35 mm tissue culture dishes coated with human placental collagen (Sigma, St. Louis, MO). Cells were patched 3–21 (mean = 7.4) days later. This method produces small to large confluent islands of cells. All patching was done on cells that were completely surrounded by other cells. All results in this paper were gathered from 15 cell-attached patches broken into 80 trials and 15 excised patches broken into 27 trials, each about two minutes duration. We used the natural variation in channel density to give patches with between 0 and 12 active channels.

Patch-clamping was carried out at 20–23°C using the cell-attached and excised configurations. Currents were recorded via an Axopatch 1C amplifier (Axon Instruments, Foster City, CA), filtered at 2000 Hz, digitized at 40 kHz (PCM-2, Medical Systems, Greenvale, NY), and stored on videotape. Electrodes were pulled from very soft glass capillaries (LA16, Dagan, Minneapolis, MN) and coated with Sylgard (Dow Corning, Midland, MI). After heat polishing, pipet resistances were 2–5 MΩ in the solutions listed below. The standard bath and cytosolic solution for excised patches (27 trials) was (in mM): NaCl 150, KCl 5, $CaCl_2$ 0.23, $MgCl_2$ 2.5, EGTA 0.5, and HEPES 10. The pH was adjusted to 7.3 with NaOH; osmolarity was adjusted to 300 mOs/l with H_2O, and free Ca^{2+} was calculated to be 100 nM. CFTR activity was maintained in excised patches with 2 mM Mg-ATP and 10 units of PKA in a 1 ml bath.

For cell-attached patches (80 trials) the standard pipette solution was (in mM): CsCl 150, $CaCl_2$ 2.5, $MgCl_2$ 2.5, and HEPES 10. Some cells were continually exposed to 10 μM forskolin for 5–60 min before and during the cell-attached recording; of 80 trials, approximately half (38 trials) were from forskolin-stimulated cells. Forskolin was obtained from Sigma and was stored at 10 mM stock solutions in ethanol at −10°C.

Except where noted, data are reported as means ± SEM. Statistical significance was assessed with the statistical packages of Microsoft Excel (*t*-test).

To calculate mean open times, records were recaptured from videotape at 25 msec/point and filtered at 10 Hz. Using Biopatch (Bio-Logic, Claix, France), all-points amplitude histograms were constructed and fit with Gaussians. Only patches with 12 or fewer channels were analyzed so that individual levels could be seen in the amplitude histograms. The area under the Gaussians was fit to a binomial distribution. The distribution was determined to be binomial if the minimum χ^2 (assuming 12 or fewer channels) produced a probability >0.9 that the distribution was binomial. The estimated number of channels in the patch (N) was the number that produced the best binomial fit. When data could not be fit with a binomial, the maximum number of simultaneous openings seen in the trial was used as N and the patch was scored as non-binomial. Idealized records were produced with a current-crossing threshold set half way between each current level and with a minimum duration of 100 msec. Use of these criteria removes the fast flicker within an open burst (Zeltwanger et al., 1999). The idealized traces show the openings (burst durations) and closings of the CFTR channel and were used for all subsequent mathematical calculations.

Definition of terms: N = Number of channels in patch, $P(o)$ = Average Current/Maximum Current, i = single-channel current.

The predicted variance for a trial is

$$\sigma^2 = P(o) \cdot (1 - P(o)) \cdot \mathrm{N} \cdot i^2 \tag{1}$$

The mean open time (*MOT*) as derived from Horn & Lange (1983) is

$$MOT = \frac{\sum_N nT_n}{E} \tag{2}$$

where n = number of channels open, T_n = duration of n channels open, E = number of events (openings).

The average current can be written as

$$I = i \cdot \frac{\sum_N nT_n}{T} \tag{3}$$

where T = total duration of trial.

Putting equations 2 and 3 together we get

$$MOT = \frac{I \cdot T}{i \cdot E} \tag{4}$$

From the definition of open-probability and equation 4 we can derive the mean closed-time (*MCT*) as

$$MCT = \frac{MOT}{P(o)} - MOT \tag{5}$$

Or the mean closed-time can be measured as

$$MCT = (\textit{Average duration of closed times}) \cdot N \tag{6}$$

The cooperativity ratio (CR) is defined as

$$CR = \frac{(P_1 \cdot P_1)}{(P_0 \cdot P_2)} \Big/ \frac{(2 \cdot N)}{(N-1)} \tag{7}$$

where P_n = probability of n channels open.

If the $CR = 1$, then the channels display a binomial histogram. If the *CR* is greater than 1 then the channels are 'heterogeneous,' ($P(o)$'s not equal), and if the *CR* is less than 1 then the channels display cooperativity.

The Horn and Lange method for estimating channel-open time assumes that the channels are identical and independent (Horn & Lange, 1983). If this condition is not met then the measured open time is a weighted average of the open times of the various non-identical or non-independent CFTR channels in the patch. The cooperativity ratio measures the deficit of single channel openings. If the channels are cooperative in some manner it is more likely that 2 channels would be open or closed together than if they were independent. The resulting deficit of single channel events produces a cooperativity ratio that would be less than 1.0. The number of events in a trial was determined by counting the number of openings and adding the number of channels open at time 0. The single-channel current was the weighted average of the distance between the peaks of the Gaussian fits. Fifty Monte Carlo simulations of 2 idealized (zero noise) independent CFTR channels (open time ~1.0 sec, closed time ~2.0 sec, when $P(o) = 0.33$), length 4800 points, (120 sec) showed cooperativity ratios that ranged between 0.5 and 2.0. This result was then used for comparison with observed distributions of CFTR activity.

RESULTS

Identity of CFTR and Behavior in Cell-attached Patches of Calu-3 Cells

The properties of CFTR channels in cell-attached patches of Calu-3 cells were briefly described by Haws et al. (1994). Figure 1*A* shows two typical trials from our experiments on cell-attached patches. CFTR channels usually appear in multi-channel patches; only 13/80 trials or 5/15 of the cell-attached patches showed single channel activity, while none of the excised patches (15) showed any single channel activity. At hyperpolarized voltages (cytoplasm negative) the single-channel currents were smaller (due to the cell-attached Cl^- asymmetry) and more flickery (Fischer & Machen, 1994; Tabcharani et al., 1990). The channel had burst durations in the range of seconds with no marked voltage dependence of the open or closed durations. The cell-attached *I-V* relation (Fig. 1*B*) was fit with a parabola with a slope of 6.7 ± 0.1 pS at +60 mV. The interpolated reversal potential was +10.9 mV, consistent with the idea of a chloride secreting epithelium, where the cell must be depolarized to reverse net chloride exit. For excised patches the *I-V* curve was linear with a slope conductance of 6.1 ± 0.2 pS. All of these values match those reported previously for CFTR. In addition, no other Cl^- channels are observed in the apical membrane of intact Calu-3 cells (Haws et al., 1994).

It has been reported that CFTR currents run down in cell-attached patches from T84 cells (Tabcharani et al., 1990). In our present experiments with cell-attached patches from Calu-3 cells, rundown was seen only in some patches, and the average current across all records

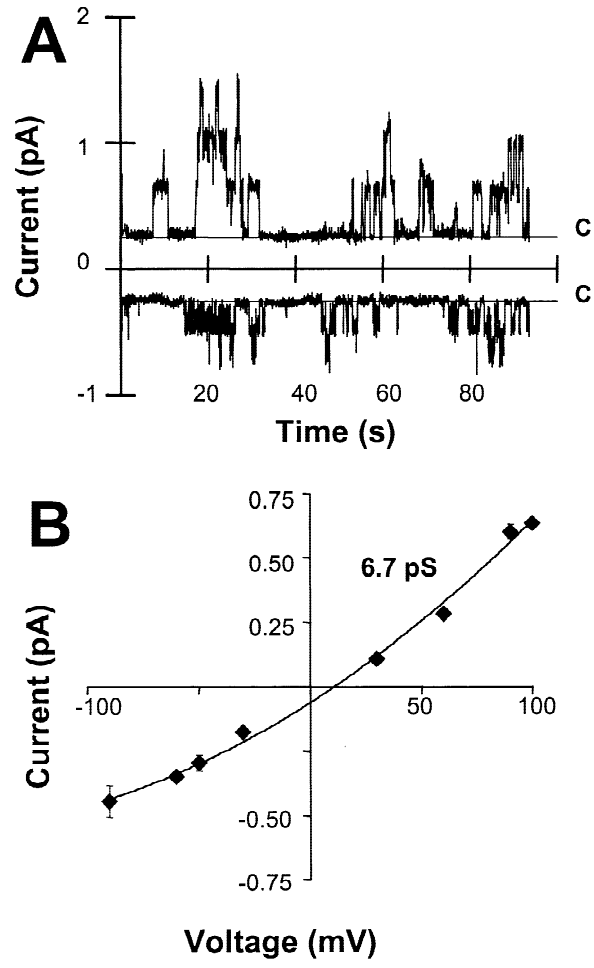
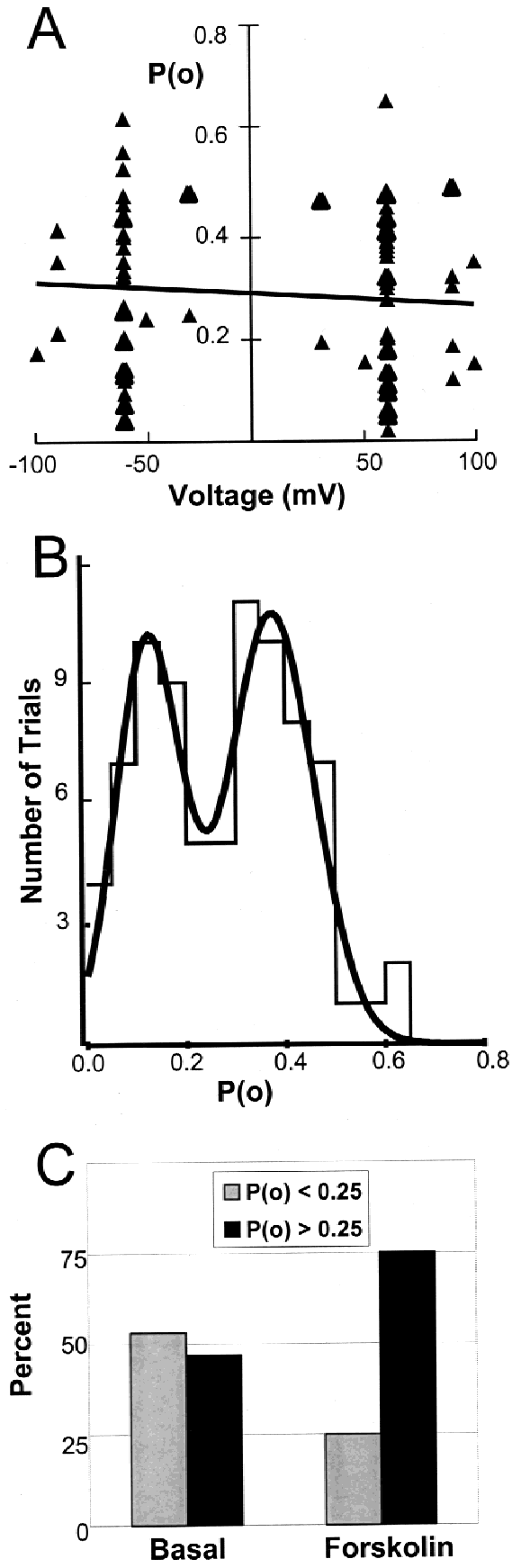


Fig. 1. (*A*) Typical single-channel records from a cell-attached patch with +60 mV (upper) and −60 mV (lower) traces shown. The channel amplitude is somewhat smaller hyperpolarized and the currents are more flickery. (*B*) Single-channel *I-V* relation for all 80 trials averaged together. The points are fit with a polynomial with a slope of 6.7 ± 0.1 pS at +60 mV. The interpolated reversal potential was +10.9 mV.

did not change with time. Using a straight line fit to each current record, the percentage change in average current per 100 seconds varied from −35% to +45%, with an average of −0.5 ± 6%.

Our results also agree with prior reports showing no effect of voltage on mean open time. In cell-attached recordings CFTR shows increased flickery-block of the channel at hyperpolarized voltages, which would reduce the measured amplitude of the unitary current and lengthen the open-duration if the CFTR channel cannot close while the channel is blocked (Neher & Steinbach, 1978). To test for voltage dependence of the open-times the calculated open-probability, $P(o)$, was plotted for each trial versus the voltage (Fig. 2*A*). There was no voltage sensitivity of the mean $P(o)$ (slope not significantly different from 0, $p > 0.3$). However, because of the large variation in the $P(o)$ at any one voltage, a volt-



age dependency could not be ruled out ($-0.8 < \Delta P(o)/V < 0.3$, 95% confidence). (With our heavy filtering, the flicker at hyperpolarized voltages is not resolved.) Flicker-induced lengthening of open time was also not seen by Fischer & Machen (1994). To reduce any flicker-induced error, all excised patches were held at +60 mV.

Open-probabilities for individual trials had a wide range. The $P(o)$ varied between 0.02 and 0.65 with the average of 0.28 ± 0.02 for cell-attached patches and 0.34 ± 0.2 for excised patches. A histogram of the cell-attached patch open-probabilities showed 2 distinct peaks with mean $P(o)$ values of 0.125 and 0.375 (Fig. 2*B*). The fit with 2 Gaussians was significantly better than a single Gaussian ($p < 0.001$). The two peaks do not reflect a difference between forskolin-stimulated and unstimulated trials, because unstimulated trials also showed two peaks. However, the percent of the patches with the higher $P(o)$ ($P(o) = 0.375$) increased from 50 to 75% after stimulation with forskolin (Fig. 2*C*). In excised patches where the PKA and ATP concentrations are fixed, the distribution of $P(o)$ is a single Gaussian centered at 0.38. The lower open-probability peak may represent channels in the low phosphorylation state (Hwang et al., 1994) or some other stable state of the CFTR molecule.

Evidence for Cooperativity: Increased Mean Open-time with Increased N

It was noted during the analysis that the $P(o)$ increased as the number of active channels increased. The mean channel number per trial was 2.2 for the lower $P(o)$ and 4.8 for the higher $P(o)$ ($p < 0.0001$). An increase in $P(o)$ with channel number was also seen by Haws et al. (Haws et al., 1994). However, because the calculation of $P(o)$ depends on N, the increase could have been an artifact of underestimating N. We therefore set out to determine the channel kinetics *independent* of N. We started by calculating the open- and closed-duration of CFTR channels in patches with 1 to 3 channels where open- and closed-times can be estimated. The results indicated that the open-times increased as the number of channels in the patch increased, but with such a small range of N the trend was not significant. We therefore used a technique

Fig. 2. (*A*) The open-probability (average current/maximum current) for all 80 cell-attached trials plotted versus the voltage of the trial. Most trials were taken at +60 and −60 mV. There is no obvious voltage dependence of the open-probability. (*B*) A histogram of the open-probabilities reveals 2 distinct peaks at $P(o) = 0.125$ and 0.375. (These peaks were independent of the bin width for bins less than 0.2). (*C*) Bar chart sowing the percent of trials with the low and high $P(o)$. Forskolin (10 μM) shifts the distribution to more trials in the high $P(o)$ state. Excised patch trials showed only 1 Gaussian centered at $P(o) = 0.38$.

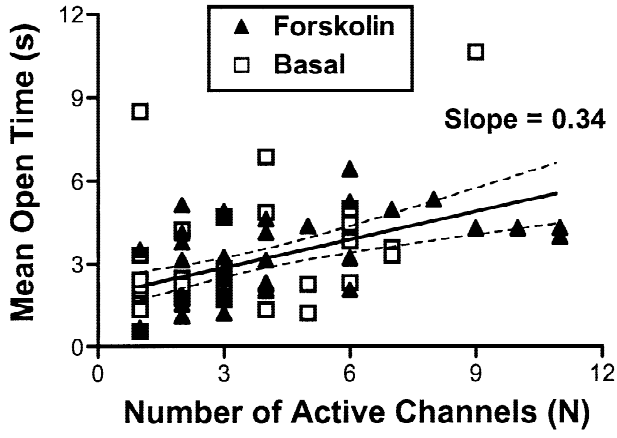


Fig. 3. (*A*) The open-duration for each of the 80 cell-attached trials only varied with the number of channels in the patch. This 34% increase in open-duration per channel is a measure of channel to channel cooperativity. The filled triangles are the data from stimulated patches (10 μM Forskolin) and the open squares are from unstimulated patches. The dotted lines represent the upper and lower 95% confidence levels. The closed time did not show a significant change with channel number (*not shown*).

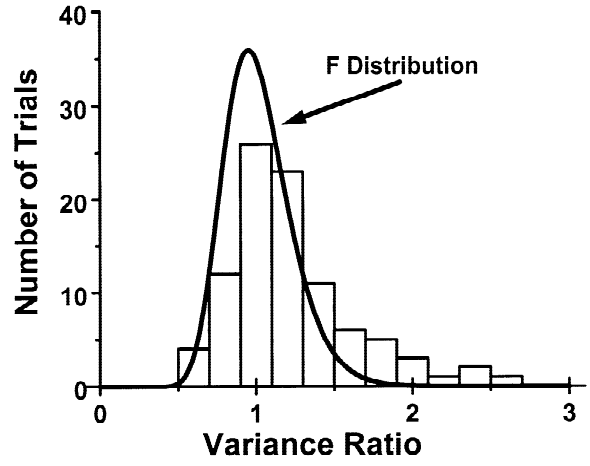


Fig. 4. The distribution of the variance ratios (variance measured/variance predicted) for all trials (cell-attached and excised) with more than 1 channel. The solid curve is the expected *F* distribution for the variance ratios ($n = 94$). Note that some trials had more variance than expected.

to measure open-times for patches with multiple channels.

Using the mathematical technique specified in the methods, we calculated the mean open-time for each trial independent of the number of channels in the cell-attached patches. From the mean open-time and the measured $P(o)$ the mean closed-time can be calculated. In Fig. 3 we plot the average open-duration versus the number of active channels seen in the cell-attached patch. A curve was fit through the data using the assumption that the opening of 1 channel changed the average open-time by a fixed proportion (i.e., linear). (For an interesting discussion of such a model *see* Liebovitch & Fischbarg, 1986). The weighted least-squares-fit line of the data was $0.34 \cdot N + 1.8$ (slope >0, $p < 0.0001$, removal of the 2 outlying points did not change the slope significantly), which corresponds to a doubling of the lifetime when ~6 channels are interacting. If the data from stimulated and unstimulated cells are separated, the same trend is seen with no significant difference in the percentage change per active channel. The estimated closed-times showed a slight decrease with channel number, but the slope was not significantly different from zero (*data not shown*). The increase in open-time with the number of active channels indicates that open CFTR channels may interact with other open CFTR channels, i.e., they display cooperativity.

Evidence for Cooperativity: Increased Variance

Another approach to look at possible cooperativity between CFTR channels is to look at the variance of the current records. If some, but not all, of the channels in a trial were shifted into a higher or lower $P(o)$ state then the channels would be heterogeneous and the noise (variance) of the current would be smaller than the average $P(o)$ predicts. This is because the variance of a population of identical channels with binomial kinetics lies on the parabola ($P(o) \cdot (1 - P(o))$), where the peak variance is at $P(o) = 0.5$ and variance goes to zero as $P(o)$ goes to 0 or 1. However, for a heterogeneous population of channels with a $P(o)$ averaged from two binomial populations having different $P(o)$ values, the weighted average of the variances will lie on the chord connecting the 2 population variances, which will always lie below the parabola.

Conversely, cooperativity can be shown to increase the variance in a signal by as much as N_L (number of linked channels)-fold. (If the cooperativity were absolute, such that all the channels opened and closed together, gating would be identical to a single channel having a single-channel current of $N \cdot i$. Since the variance is proportional to i^2 and N, the net effect is to increase the variance by N_L).

Analysis of all 94 trials (with more than 1 channel) revealed variances that were roughly within a factor of 2 of the predicted variance. The variance distribution of the data is predicted by a scaled *F* (variance ratio) distribution as seen in Fig. 4. There is no evidence for hidden, low variance heterogeneous channels by this analysis. However, there is evidence for a lack of low variance trials and an increase in high variance trials, as predicted by cooperativity. The deviation of the data from the *F* distribution is significant (Kolomgrov-Smirnov $p < 0.001$).

Evidence of Cooperativity: Measuring Cooperativity Ratios

We further evaluated cooperativity in CFTR gating by using a third, independent measure of cooperativity devised by Manivannan et al. (1992). Because the number of channels in our patches varies, we refined their cooperativity measure into a *cooperativity ratio* that considers the number of channels present (Manivannan et al. 1999). To calibrate this measure, we performed 50 simulations of gating by 2 independent CFTR-like channels. In this simulation the cooperativity ratio was never less than 0.5 nor greater than 2.0. In the 16 experimental trials that had two channels, 2 trials had a ratio less than 0.5 and 5 trials had a ratio greater than 2.0. The excess of trials with a ratio >2.0 implies that the channels have non-identical open-probabilities. The trials with ratios <0.5 imply that some, if not all, of the channels are gating cooperatively. Expanding the analysis to include all trials with more than 2 channels revealed 6 additional trials with a cooperativity ratio of <0.5. The 8 cooperative trials each occurred once in 8 separate patches, 2 unstimulated cell-attached, 4 stimulated cell-attached, and 2 excised.

A cooperativity ratio >2.0 occurs when the open probabilities of the channels in the patch differ or the $P(o)$ changes with time. A cooperativity ratio >2.0 was observed in 21 of 94 trials. As with cooperativity, this evidence for heterogeneity among the channel-open probabilities is significantly beyond the level expected by chance for channels displaying purely binomial statistics (χ^2 test, $p < 0.0001$). This kind of activity is important for two reasons. First, it emphasizes that CFTR channels are not well fit with assumptions of independent, equivalent channel statistics. More importantly, this heterogeneity in CFTR channel $P(o)$ masks the ability to measure cooperativity using the cooperativity ratio. We have used the name 'heterogeneous' to describe this type of channel activity. The 21 heterogeneous trials occurred in 14 patches, some of which showed cooperativity at a different time.

The cooperative and heterogeneous cell-attached trials are graphed separately in Fig. 5. The normalized percentage increase in channel-open time with channel number increase from 34% to 95 ± 11% for the cooperative patches ($p < 0.05$, Fig. 5*A*) and 56 ± 27% for the heterogeneous trials ($p > 0.05$, Fig. 5*B*). The average N's for these sets of patches were not significantly different. However, as expected, the variance of the cooperative patches was significantly higher than the variance of the heterogeneous patches ($p < 0.05$). Each group included both stimulated and unstimulated patches, so that neither type of behavior can be attributed solely to stimulation. For the 2 excised patches showing cooperativity the open-time increased 2.1-fold while the number of channels increased 2.4-fold (slope 88%). The

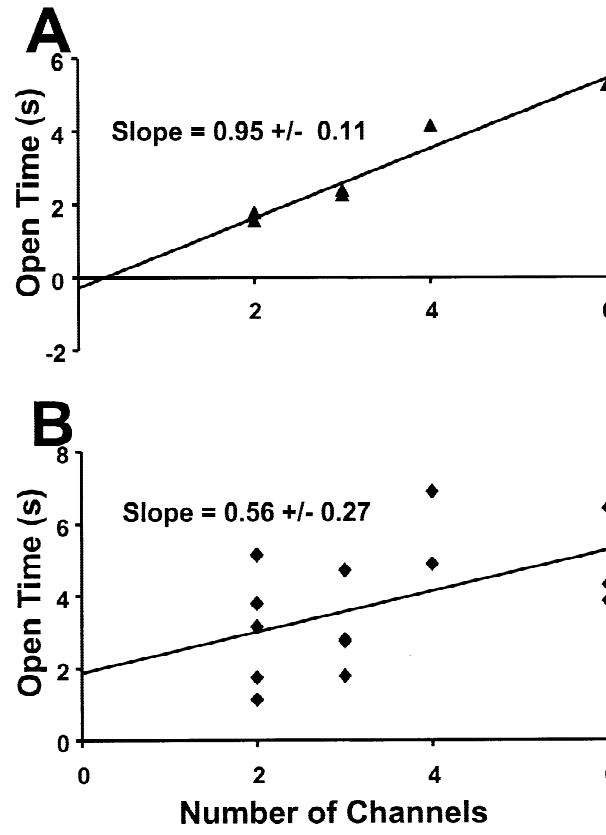


Fig. 5. (*A*) The open-times of the 'cooperative' patches increase dramatically with channel number (slope 0.95 ± 0.11); for the 2 cooperative trials in excised patches the slope was 0.88. (*B*) The open-time of the 'heterogeneous' cell-attached patches did not increase significantly with channel number (0.56 ± 0.27).

slope of the open time *vs.* N is also dependent on the correct estimation of N. To check the accuracy of our estimate, we compared the closed-times measured two separate ways (Eqn. *5* & *6*). The two sets of closed times were not significantly different (*t*-test), implying that our estimate of N was accurate within ± 10% (*data not shown*).

Thus, the cooperativity ratio suggested by the work of Manivannan et al. (1992) provides a third measure of cooperativity between CFTR channels. Because cooperative, heterogeneous and binomial channel behavior can occur in the same patch at different times, an average of all activity will tend to be indistinguishable from binomial activity. This raises the intriguing possibility that binomial statistics are not the normative behavior of populations of CFTR channels, but instead arises as a consequence of averaging between cooperative and heterogeneous activity (*see* Discussion). Note that because all three forms of activity (binomial, cooperative and heterogeneity) occur within a single patch, the factors that gave rise to cooperative and heterogeneous activity must be dynamic.

Discussion

What Did We Observe?

CFTR channels are complexly regulated, therefore complex kinetics are expected. In multiple channel patches, we documented three aspects of channel gating that depart from predictions based on the independent gating of channels with uniform average gating properties. First, the mean open-time increased as channel number increased (Fig. 3). Second, the distribution of variances of each trial indicated an excess of trials with higher variance (Fig. 4). Third, estimates of binomial gating (based on cooperativity ratio) revealed a distribution that departed significantly from the expected distribution, indicating the presence of a subset of trials displaying cooperativity and a subset displaying heterogeneity as defined in Methods, (definition of terms). The departures from the expected cooperativity ratio distribution were highly significant ($p << 0.001$). (Note that in the paper by Keleshian et al. (2000), only 2 of the 4 data sets in table 2 pass our requirement of a cooperativity ratio less than 0.5, although the authors claim that all 4 data sets show cooperativity. Thus, our use of a cooperativity ratio less than 0.5 is fairly stringent.)

From these results we deduce that: (*i*) CFTR channels changed their open-probabilities over time in our experiments, (*ii*) different channels in the same patch can have different open-probabilities averaged over the time course of a 2 minute trial, and (*iii*) as the number of active channels in a patch increases, the open-duration of the channels also increases, but the closed times do not decrease. These departures from binomial statistics occurred in both unstimulated and constantly stimulated cells, and in excised patches exposed to a constant concentration of ATP and PKA.

It is important to remember that the formula for the mean open time assumes that the channels are homogeneous. If the channels are heterogeneous the mean open-time is not the open-time of any specific subset of channels, but the weighted average of all the different channel populations. Only in the pure homogeneous trials is the estimated mean open-time expected to equal the actual mean open-time for the individual channel. This is the case in the 8 cooperative trials. We expect all channels in each of these trials to be identical because there is no evidence of heterogeneity in these 8 trials.

A more precise test for cooperativity compares the average duration of n open channels when the preceding state was $n + 1$ open channels with the average duration when the preceding state was $n - 1$ open channels (Yeramian, Trautmann & Claverie, 1986). The deviations from 1.0 are not large and thousands of events are needed for an accurate determination. This analysis can only be applied to homogeneous channels. We can only be sure that the 8 trials that show cooperativity do not have a significant contamination from heterogeneity. Unfortunately, summing all 8 trials yielded only ~300 transitions to the single open level, which is not enough data for this type of analysis. Therefore, we chose to use the Manivannan method as a means to screen trials for cooperativity and further analysis.

How Do We Interpret These Results?

The conclusion that the open-probabilities of channels in the patch are not identical or may vary with time is not remarkable. Indeed, the different $P(o)$s might simply indicate temporal and spatial heterogeneity in the complex processes that control CFTR channel gating in these intact, confluent cells. With regard to temporal heterogeneity, short circuit current (I_{SC}) studies of confluent monolayers of Calu-3 cells often reveal marked oscillations in I_{SC} (caused by variations in intracellular Ca^{2+}) with time courses of minutes from maximum to minimum I_{SC} (Moon et al., 1997, Shen et al., 1994). Given such coordinated oscillations across millions of cells, local oscillations of any intracellular messenger might be expected to be common. With regard to spatial heterogeneity, there is increasing evidence for specific targeting of signaling pathways that were once thought to be spatially uniform (Pawson & Scott, 1997). Thus, although temporal/spatial heterogeneity might be expected in any fine-grain analysis, the apparent cooperativity result is unexpected and is of potential importance. A strong, direct interpretation of the finding that the open duration increases with the number of active channels is that an open CFTR channel can stabilize the open conformation of an adjacent channel.

Because the number of patches which showed only cooperativity is small ($n = 8$), we considered two alternative hypotheses to explain our results.

One interpretation is that some activation factor (such as PKC levels) rises within the cell to activate quiescent channels and increase the channel-open time of all the channels. This would account for channel-open time increasing with channel number. However, we consider this explanation unlikely. The activation (or inactivation) *must* occur within a trial to produce the excess variance seen and a cooperativity ratio <0.5. To explain our results the factor must more than double the number of channels in a trial while doubling their open times. A simple doubling is *not* enough to produce either a variance ratio >2.0 or a cooperativity ratio <0.5, but would produce a rundown/runup of up to 400%. Such large variations in current were not seen; rundown/runup ranged only between −35% to +45%. It also seems unlikely that under maximum stimulation with forskolin or activation by ATP and PKA there would be any quiescent channels (Yamazaki et al., 1999). (While PKC can

cause the activation of quiescent channels (Jia, Mathews & Hanrahan, 1997) the reported increase in current ~30% (Winpenny et al., 1995) is not sufficient to account for our data.) Finally, this hypothetical activation factor must also exist in excised patches, and that seems unlikely.

The magnitude of the cooperative interaction between CFTR molecules can be estimated with a few assumptions. 1) In the 8/94 cooperative trials, all channels in the patch were in the cooperative mode. 2) Channels can exist in 2 states: cooperative or independent. Since the average number of channels in a patch was 4, then $(8/94) = c^4$ and $c \approx 0.5$, where c is the probability a channel is in the cooperative mode. Thus the probability of 2 channels interacting is 25%.

What Mechanisms Could Account for our Interpretation?

A mechanism whereby CFTR molecules can influence other molecules has been proposed by Short et al. (1998). CFTR has a PDZ domain on the C-terminus that can bind EBP50 (NHE-RF) and other apical proteins. This presents the possibility that 2 CFTR molecules can be linked via other proteins and hence physically interact. Wang et al. (2000) recently showed that CFTR molecules can interact by binding to CAP70 PDZ binding domains. When 2 CFTR molecules were bound to one CAP70 molecule the open time increased 3 to 4 fold for both channels. If the CFTR-CAP70 interaction is the basis for cooperativity, our data suggest that in Calu-3 cells the probability of a CAP70 molecule binding 2 CFTR molecules is 25% and that the interaction can last at least 120 sec. It may be that CFTR molecules are somewhat free to diffuse in the membrane and if 2 CFTR channels form a transient multimer with CAP70, this may serve to stabilize the open states (*see* discussion in Keleshian et al., 2000) for other channels interacting as dimers). Other physical and electrophysiological evidence for CFTR dimers exists (Eskandari et al., 1998; Sheppard et al., 1994; Zerhusen et al., 1999), but there has been no suggestion of CFTR multimers. If dimer formation completely explained the increased open time with increased N, then the relation between N and open-time should saturate when all channels are dimerized and maximally open. Our data in Fig. 5 indicate that the relation is linear up to at least 5 channels so the saturation occurs at 10 or more channels. In fact, inspection of the forskolin-stimulated data in Fig. 3 shows what appears to be a saturation at higher Ns, consistent with a dimerization hypothesis.

Another recent paper (Raghuram, Mak & Foskett, 2001) showed a marked increase in CFTR $P(o)$ following addition of nM quantities of bivalent PDZ-domain peptides, yet they concluded that the channels were independent because binomial statistics were observed both before and after addition of the peptide. However, their model proposes that CFTR-PDZ complexes exist in discrete, long-lived (>100 sec) states with at least a 2-fold difference in $P(o)$ between the least active and most active state. (They propose 4 states.) Thus, according to their model, in limited-duration (300 sec) multi-CFTR channel patches exposed to PDZ peptides the channels *must* show heterogeneous gating, yet they observed binomial statistics in 5 multichannel patches. As discussed previously, cooperativity may be masked when channels in a patch have unequal open probabilities, so that a patch containing both highly cooperative and highly heterogeneous channels may be indistinguishable from a patch with homogeneous, independently gating channels. While it is possible that their model of discrete states is incorrect, we favor an interpretation that preserves a discrete state model, and explains the apparent binomial statistics as a result of the competing effects of heterogeneous gating and cooperativity.

If We Provisionally Accept Cooperativity, What are the Consequences?

Cooperativity among CFTR channels means that channel-open probability is not simply a function of phosphorylation level or ATP levels, but also depends upon channel density. As channel density increases, cooperativity predicts a steeper stimulation response curve and a higher average $P(o)$ (approaching one if more than 16 channels capable of interacting, as has been observed experimentally (Fischer et al., 1992; Fischer & Machen, 1994). With very high channel densities, cooperativity might lead CFTR to be active at resting levels of $[cAMP]_i$. The highest levels of endogenous CFTR are observed in the native sweat duct (Quinton, 1986) and in Calu-3 cells (Finkbeiner, Carrier & Teresi, 1993), and in both cases CFTR appears to be active in unstimulated cells (Moon et al., 1997, Quinton, 1986; but *see* Devor et al., 1999). When cells are transfected with exogenous CFTR, higher expression levels were associated with basal activity of CFTR (Stutts et al., 1993).

Conversely, at sufficiently low channel densities, cooperativity may be eliminated with corresponding decreases in the dose-response relation and in maximum $P(o)$. As proposed by Li et al. (1993), the low $P(o)$ of ΔF508 CFTR observed in many systems may be due to its low density in the membrane rather than to a faulty intrinsic gating mechanism. As they showed, high levels of plasma membrane ΔF508 CFTR can be achieved by baculovirus expression in insect cells, in such circumstances ΔF508 CFTR gating appeared to be like wild type CFTR (Le et al., 1993).

Other Aspects of the Data

The histogram of the calculated $P(o)$ of all 80 trials shows two peaks. The origin of the two peaks is un-

known, but they do not depend on whether the cell was stimulated with forskolin or not. We separately analyzed channel kinetics for the low and high $P(o)$ trials, but because $P(o)$ is primarily determined by CFTR phosphorylation and cytosolic ATP levels, we considered explanations in those terms. Some single-channel data have been interpreted to mean that CFTR has two preferred phosphorylation states that correlate with $P(o)$ (Hwang et al., 1994), with the higher phosphorylation state being characterized by longer open times. Thus, if the two peaks in our study arise from two phosphorylation states, we would expect the higher $P(o)$ to result from longer open-times rather than more frequent openings. Comparing data from low $P(o)$ (<0.25) to high $P(o)$ (>0.25) trials, we found that the mean open-time increased from 2.1 sec to 3.9 sec. Conversely, higher $P(o)$ can also result from higher ATP levels, but in this case there should be more frequent openings (Venglarik et al., 1994). Comparing data from low $P(o)$ to high $P(o)$ trials, we found that the mean closed time decreased from 17.2 sec to 6.1 sec. Both results were highly significant ($p < 0.001$). Thus the two preferred $P(o)$ states do not result from a single change in either open or closed times.

In conclusion, our data indicate that CFTR channel kinetics do not uniformly obey binomial statistics. Instead, small, but highly significant subsets of channel activity display unequal open-probabilities or correlations among open-times. Although the preponderance of channel kinetics in multiple channel patches were regarded as binomial, the two departures from binomial statistics give the appearance of binomial behavior when they are averaged, raising the possibility that the small subsets of non-binomial kinetics are actually normative. The probability of a trial being heterogeneous is at least 22% (21/94). However, it should be noted that any patch where only some of the channels are interacting in a cooperative manner must also be heterogeneous because there are at least 2 sets of channels with different gating properties. We do not presently see how to distinguish a patch where only some of the channels are interacting cooperatively (thus the channels are heterogeneous) from the conventional interpretation that the patch has identical independent channels. We have estimated the probability of 2 channels showing cooperative gating as 25%.

Regardless of the relative proportion of channel activity in each kinetic state, the evidence for cooperativity indicates that CFTR channels can influence one another. Masking by 'heterogeneous' (i.e., unequal $P(o)$) kinetics causes cooperativity to be underestimated for CFTR channels. The role of cooperativity may be crucial when attempting to explain CFTR behavior in different organs, species, and disease states, where natural expression levels of CFTR in the plasma membrane can vary by manyfold.

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