Evidence for Cooperativity Between Nicotinic Acetylcholine Receptors in Patch Clamp Records

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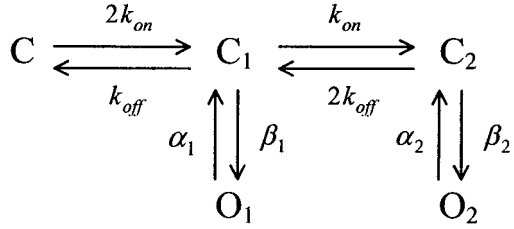
ABSTRACT It is often assumed that ion channels in cell membrane patches gate independently. However, in the present study nicotinic receptor patch clamp data obtained in cell-attached mode from embryonic chick myotubes suggest that the distribution of steady-state probabilities for conductance multiples arising from concurrent channel openings may not be binomial. In patches where up to four active channels were observed, the probabilities of two or more concurrent openings were greater than expected, suggesting positive cooperativity. For the case of two active channels, we extended the analysis by assuming that 1) individual receptors (not necessarily identical) could be modeled by a five-state (three closed and two open) continuous-time Markov process with equal agonist binding affinity at two recognition sites, and 2) cooperativity between channels could occur through instantaneous changes in specific transition rates in one channel following a change in conductance state of the neighboring channel. This allowed calculation of open and closed sojourn time density functions for either channel conditional on the neighboring channel being open or closed. Simulation studies of two channel systems, with channels being either independent or cooperative, nonidentical or identical, supported the discriminatory power of the optimization algorithm. The experimental results suggested that individual acetylcholine receptors were kinetically identical and that the open state of one channel increased the probability of opening of its neighbor.

INTRODUCTION

The nicotinic acetylcholine receptor is a single pore membrane-bound ion channel formed from five protein subunits, with normal gating controlled by the binding of acetylcholine to two agonist recognition sites. Stochastic modeling of the single receptor kinetics is often based on a five-state continuous-time Markov model with equal affinity at both binding sites (Ball and Sansom, 1989), as follows,



Scheme 1

Here, C, C_1, and C_2 represent unliganded, mono-, and bi-liganded closed states of the receptor respectively; O_1 and O_2 are open channel states; and the transition parameter k_{on} is proportional to the concentration of acetylcholine.

When the membrane patch contains more than one concurrently active channel, interpretation of observed kinetics requires more complex modeling. If the channels are identical, this can be achieved by adapting the Markov model to take into account joint states across receptors. For example, in the case of two channels, each modeled as in Scheme 1, the expanded Markov process will have 25 states consisting of nine jointly closed (both receptors closed), 12 states where only one receptor is open, and four jointly open. However, provided the channels behave independently, it is possible to derive many single channel statistics without considering the expanded Markov process (see Yeo et al., 1989 or Dabrowski et al., 1990).

While the assumption of independence of ion channel activity is widespread, it may not always be appropriate (Iwasa et al., 1986). In the case of nicotinic acetylcholine receptors, channel kinetics in *Xenopus* muscle cells appear affected by receptor clustering (Young and Poo, 1983) and there is evidence of cooperative interaction (Schindler et al., 1984; Yeramian et al., 1986), though possible mechanisms remain unclear. Cooperativity can be incorporated into kinetic models of multiple channel systems by assuming that certain transitions in one channel can cause instantaneous changes in specified transition rates of neighboring channels. Such a system can still be modeled as an expanded continuous-time Markov process (Blunck et al., 1998), and analyzed using conventional methods (Colquhoun and Hawkes, 1981; Ball et al., 1997). In the simple case where the patch contains only two active identical channels, separate open and closed time densities for one channel, given the neighboring channel is either open or closed, can sometimes be derived from the experimental recording without consideration of the expanded Markov process (Keleshian et al., 1994). If the channels are independent, these conditional densities will be identical; conversely, differences in

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either the open or the closed time conditional densities suggest cooperativity.

The aims of the present study were twofold. First, to examine steady-state patch clamp data from concurrently active nicotinic receptors for preliminary evidence of interdependence between channels by comparing experimental data with predictions of the binomial distribution. Second, in those cases where patches contained only two active channels, the two open time and the two closed time conditional densities for each channel would be used to decide more rigorously whether channels were independent and identical.

METHODS

Cell culture

Briefly (see Le Dain et al., 1991 for further details), thigh muscles from 10-day-old chick embryos were dissociated in 0.25% trypsin for 15 min at 37°C in divalent cation-free phosphate buffered saline. The resulting solution was centrifuged at 500 × *g*, the supernatant discarded, and the cells resuspended in nutrient medium at a concentration of ~4×10^5 cells/ml. The nutrient medium consisted of Minimum Essential Medium (ICN Pharmaceuticals, Costa Mesa, CA) and also included 8 mM $NaHCO_3$, 5% Foetal Calf Serum (CSL, Melbourne, Australia), 5% Horse Serum (CSL), 2% chicken embryo extract, 75 U/ml penicillin, and 45 μg/ml streptomycin adjusted to a pH of 7.4. Five milliliters of the cell suspension (containing ~2×10^6 cells) were added to each plastic petri dish, which contained a gelatine-coated glass coverslip, and then incubated at 37°C in a humidified environment in the presence of 2% CO_2. Subsequently, every third day the medium was replaced with fresh medium containing 1 μM cytosine arabinoside (Sigma, St. Louis, MO).

Data recording

On day 7–8 in vitro, after myoblasts had grown sufficiently and fused to form myotubes, the coverslip was placed in a simple buffer solution (NaCl 150 mM, KCl 5 mM, $CaCl_2$ 2 mM, $MgCl_2$ 1 mM, HEPES 10 mM, and tetrodotoxin 0.1 μM) suitable for patch clamp recording at room temperature. Borosilicate glass micropipettes (tip resistance 2–5 MΩ) coated with Sylgard 184 (Dow Corning, Midland, MI) were filled with buffer solution containing 0.2 μM acetylcholine (Sigma) and used to record single channel currents in the cell-attached configuration, with a hyperpolarizing voltage of +20 mV routinely applied to improve signal-to-noise ratio. Seals were usually better than 10 GΩ. Data were filtered at 3 kHz (8-pole Bessel filter), recorded on FM tape and then digitized off-line at 20 kHz with a 12-bit analog-to-digital converter for further analysis.

Signal extraction

Digitized data were divided into sequential frames of 9984 points and visually inspected. Some frames with excess noise (e.g., transient seal breakdown) or unstable baseline were deleted, while still leaving uninterrupted sojourns as long as several thousand milliseconds for analysis. Any remaining baseline shift between frames was corrected by superimposing amplitude histograms from each frame. Single channel current was then estimated from the amplitude histogram of each data set. Data were idealized using the forward and backward algorithms (Chung et al., 1990) and a hidden Markov model, with levels for concurrent activity corresponding to integer multiples of the estimated single channel current. The noise standard deviation for all conductance levels was estimated from sections of recording containing no visible channel openings.

Binomial distribution

From the idealized trace, the maximum number of concurrent openings was used as an estimate of the number of active channels (N) in the patch. Assuming records were at steady state and channels were independent and identical, the probability of a single channel being open (p_o) was estimated by minimizing the sum of squared differences between the observed probabilities (P_{obs}) of occupancy of the various conductance levels and the expected probabilities (P_{exp}) obtained from the binomial distribution. Any significant deviation of the ratio P_{obs}/P_{exp} from unity would suggest cooperativity between the channels, conditional on appropriateness of the above assumptions. To confirm that recordings were made under steady-state conditions, each data set was partitioned into consecutive time segments of 5 s duration and the probability of occupancy of each conductance level was inspected as a function of recording time for non-steady-state trends. The possibility of the channels being nonidentical (having different open probabilities) was examined analytically by visualizing the changes in the ratio P_{obs}/P_{exp}, where P_{exp} was calculated assuming independent and identical channels. The assumption that the number of active channels in the patch was equivalent to the maximum number of concurrent openings was examined by simulation studies. Simulated data with 2, 3, and 4 independent and identical channels were produced by sampling the open and closed time density functions computed from records obtained under identical experimental conditions and having a single active acetylcholine receptor (Liu and Madsen, 1996). The simulations were used to compute the probability of observing the maximum number of concurrent openings as a function of simulated data length. Although the present study focused on possible interactions between separate nicotinic receptors, it should be noted that data from multimeric channels with equivalent conductance substates or multipores represent a parallel analytical problem, and many binomial-based studies addressing the question of independence have been reported (Krouse et al., 1986; Queyroy and Verdetti, 1992; Chen and DeHaan, 1992; Hayman and Ashley, 1993).

Conditional density functions

In data sets where only two concurrent openings were observed, and assuming that the channels could be nonidentical and cooperative with kinetics as in Scheme 1, the two-channel system was modeled as an expanded Markov chain. The four conditional density functions for each receptor were obtained by optimizing the appropriate parameters to conjointly maximize the fit to the observed steady-state probabilities and the likelihood of the densities of the six possible sojourn types (see Fig. 1). The conditional densities for channel 1, for example, are denoted by $f^{(1)}_{Y|C}(t)$, $f^{(1)}_{Y|O}(t)$, $f^{(1)}_{X|C}(t)$, and $f^{(1)}_{X|O}(t)$ where Y (respectively X) is the random variable

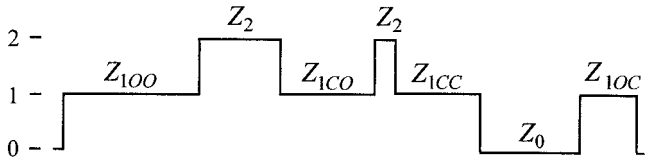

FIGURE 1 A segment of idealized recording illustrating notation for all possible sojourn types observed in a patch with two active channels. Conductance levels 0, 1, and 2 correspond to neither, one, or both channels open, respectively. Random variables Z_0 and Z_2 denote, respectively, a sojourn time at level 0 and 2; while Z_{1OC}, for example, corresponds to a sojourn time at level 1 that begins with an opening (O) and ends with a closure (C).

associated with single channel closed (open) times, and $f^{(1)}_{Y|C}(t)$ would be the density function of closed times in channel 1 given that channel 2 is closed.

The reliability of conditional density function estimation was examined with simulated data by considering all four possible cases where the two channels could be independent or nonindependent and either kinetically identical or nonidentical. The number of sojourns in these simulations was comparable to the number observed in the experimental data. Scheme 1 was assumed to describe single channel kinetics with transition parameters (ms^{-1}) $k_{on} = 0.02$, $k_{off} = 7.69$, $\beta_1 = 3.1$, $\beta_2 = 31.0$, $\alpha_1 = 9.687$, and $\alpha_2 = 0.9687$ (Ball and Sansom, 1989). Nonidentical behavior in channel kinetics was studied by assigning different opening transition rates to the two channels, whereas cooperativity between the channels was modeled by increasing the opening transition rate in either channel when its neighbor opens.

The following notation is used throughout the paper: transition rates in channel 1 given channel 2 is open are denoted by $k'^{(1)}_{on}$, $k'^{(1)}_{off}$, $\beta'^{(1)}_1$, $\beta'^{(1)}_2$, $\alpha'^{(1)}_1$, and $\alpha'^{(1)}_2$, while $k^{(1)}_{on}$, $k^{(1)}_{off}$, $\beta^{(1)}_1$, $\beta^{(1)}_2$, $\alpha^{(1)}_1$, and $\alpha^{(1)}_2$ are the corresponding rates if channel 2 is closed. Similarly, transition rates for channel 2 are denoted with a superscript (2). Transition rates for the simulation studies are given in Table 1. In the case of identical channels, corresponding parameters are equivalent; for example, $k'^{(1)}_{on} = k'^{(2)}_{on}$, and they can then be collectively denoted k'_{on}. To ensure microscopic reversibility of the expanded Markov model, the following relations must hold:

$$k'^{(1)}_{off} k^{(1)}_{on} = k^{(1)}_{off} k'^{(1)}_{on}$$

$$k'^{(2)}_{off} k^{(2)}_{on} = k^{(2)}_{off} k'^{(2)}_{on}$$

$$\alpha'^{(1)}_1 \beta^{(1)}_1 \alpha^{(2)}_2 \beta'^{(2)}_2 = \alpha^{(1)}_1 \beta'^{(1)}_1 \alpha'^{(2)}_2 \beta^{(2)}_2$$

$$\alpha'^{(2)}_1 \beta^{(2)}_1 \alpha^{(1)}_2 \beta'^{(1)}_2 = \alpha^{(2)}_1 \beta'^{(2)}_1 \alpha'^{(1)}_2 \beta^{(1)}_2$$

$$\alpha'^{(1)}_2 \beta^{(1)}_2 \alpha^{(2)}_2 \beta'^{(2)}_2 = \alpha^{(1)}_2 \beta'^{(1)}_2 \alpha'^{(2)}_2 \beta^{(2)}_2$$

$$\alpha'^{(2)}_1 \beta^{(2)}_1 \alpha^{(1)}_1 \beta'^{(1)}_1 = \alpha^{(2)}_1 \beta'^{(2)}_1 \alpha'^{(1)}_1 \beta^{(1)}_1$$

For identical channels, $k'_{off} k_{on} = k_{off} k'_{on}$ and $\alpha'_1 \beta_1 \alpha_2 \beta'_2 = \alpha_1 \beta'_1 \alpha'_2 \beta_2$ are sufficient for microscopic reversibility, and each pair of conditional densities can then be related using convolution to obtain functions $K_C(t)$ and $K_O(t)$ that characterize and quantify dependence between channels (Keleshian et al., 1994). These are respectively defined as the integral of $k_C(t)$ and $k_O(t)$ where $f_{Y|O}(t) = k_C(t)*f_{Y|C}(t)$ and $f_{X|O}(t) = k_O(t)*f_{X|C}(t)$, the symbol * denoting convolution.

RESULTS

Acetylcholine (0.2 μM) induced inward currents in cell-attached recording mode, and these channels were not seen in the absence of acetylcholine or when 200 nM α-bungarotoxin was present in the recording pipette (Liu and Madsen, 1996). Most records contained only single level openings. From these, single channel conductance was estimated to be ~35 pS. Gating kinetics with mean open time constants of 0.53 and 16.7 ms were also obtained, in substantial agreement with earlier studies (for example, 0.7 and 17.9 ms found in denervated rat muscle cells by Gage and McKinnon, 1985). These channels in cultured chick myotubes, studied at days 7–8 in vitro, formed a homogeneous class of nicotinic receptors of the embryonic type with coefficients of variation in the fast and slow open time constants of 23% and 13% ($n = 8$ experiments) respectively. The adult-type receptor, with a slow open time constant of 5.7 ms, was not seen until 12–14 days in vitro (Liu and Madsen, 1996).

Some data sets, collected under conditions of constant acetylcholine concentration (0.2 μM), contained two or more concurrently active channels, due most likely to an increased number of receptors in particular patches. Four of these with an acceptable signal-to-noise ratio and an absence of any rundown in channel activity were selected for detailed study of possible interaction effects (see Table 2). All these data sets contained transitions between open channel conductance levels where, at least visually, two or more channels seemed to open or close simultaneously (Fig. 2). This phenomenon would be extremely rare if individual channels were acting independently, given the recording system dead time (~100 μs). Each data set was examined for steady-state conditions by partitioning the records into consecutive segments of 5-s duration as described. Differences between the probability of occupancy of each conductance level across segments were well within expected sampling variation, with no consistent non-steady-state trends (see Fig. 3 for results from one data set). With the

TABLE 1 Transition parameters (ms^{-1}) of the expanded two-channel Markov process used for simulations with individual channels modeled as in Scheme 1

Transition Parameters	Cooperative Nonidentical	Cooperative Identical	Independent Nonidentical	Independent Identical
$k^{(1)}_{on}$, $k'^{(1)}_{on}$, $k^{(2)}_{on}$, $k'^{(2)}_{on}$	0.02	0.02	0.02	0.02
$k^{(1)}_{off}$, $k'^{(1)}_{off}$, $k^{(2)}_{off}$, $k'^{(2)}_{off}$	7.6923	7.6923	7.6923	7.6923
$\alpha^{(1)}_1$, $\alpha'^{(1)}_1$, $\alpha^{(2)}_1$, $\alpha'^{(2)}_1$	9.6875	9.6875	9.6875	9.6875
$\alpha^{(1)}_2$, $\alpha'^{(1)}_2$, $\alpha^{(2)}_2$, $\alpha'^{(2)}_2$	0.96875	0.96875	0.96875	0.96875
$\beta^{(1)}_1$	2.0667	3.1	2.0667	3.1
$\beta'^{(1)}_1$	6.2	9.3	2.0667	3.1
$\beta^{(1)}_2$	20.667	31.0	20.667	31.0
$\beta'^{(1)}_2$	62.0	93.0	20.667	31.0
$\beta^{(2)}_1$	6.2	3.1	6.2	3.1
$\beta'^{(2)}_1$	18.6	9.3	6.2	3.1
$\beta^{(2)}_2$	62.0	31.0	62.0	31.0
$\beta'^{(2)}_2$	186.0	93.0	62.0	31.0

TABLE 2 Observed steady-state probabilities and ratio of observed to predicted steady-state probabilities for different conductance levels in recordings exhibiting concurrent channel activity

Data Set	Maximum Number of Openings	Conductance Level		
		0	1	≥2
1	3	0.9512*	0.0476	0.0012
		1.00†	0.99	1.53
		(2568)‡	(2766)	(159)
2	2	0.9869	0.0130	0.0001
		1.00	1.00	2.25
		(407)	(493)	(6)
3	2	0.9836	0.0162	0.0002
		1.00	0.99	3.75
		(1121)	(1196)	(23)
4	4	0.8741	0.1173	0.0086
		1.00	0.99	1.40
		(694)	(870)	(131)

Each cell contains:
*Observed probability (computed from all digitized points in each data set);
†Ratio of observed probability to expected binomial probability; and
‡Number of sojourns.

assumption that the channels were independent and identical, the probability of a single channel being open (p_o) was estimated using the binomial theorem, and the resulting predicted probabilities at levels 0, 1, and 2 or more were compared to the observed steady-state probabilities (Table 2). Experimentally observed probabilities of multiple openings were greater than those predicted, irrespective of whether two or more channels were observed in the patch. This suggests positive cooperativity between channels, provided the channels were identical and the number of channels in the patch had been correctly estimated.

These two qualifications were then explored analytically and by simulation, respectively. First, if one assumes independent and identical channels in a case where the channels are actually not identical, the ratio P_{obs}/P_{exp} of multiple openings is less than unity, decreasing as the steady-state open probabilities of the channels diverge. A three-channel case with probability 0.89 of the patch not conducting (comparable to experimental data) is shown in Fig. 4. The results are clearly opposite to the data given in Table 2. Secondly, although the maximum number of concurrent openings observed in an experimental recording constitutes the best estimate of the number (N) of active channels in the patch when $N \leq 4$ (Horn, 1991), the possibility of the results (P_{obs}/P_{exp}) in Table 2 being misleading because of incorrect assignment of N was examined. Fig. 5 shows the probability of observing at least one occurrence of all channels being concurrently open as a function of simulated data length in cases with two to four independent and identical channels. The data were generated by sampling the open and closed time density functions obtained from patches

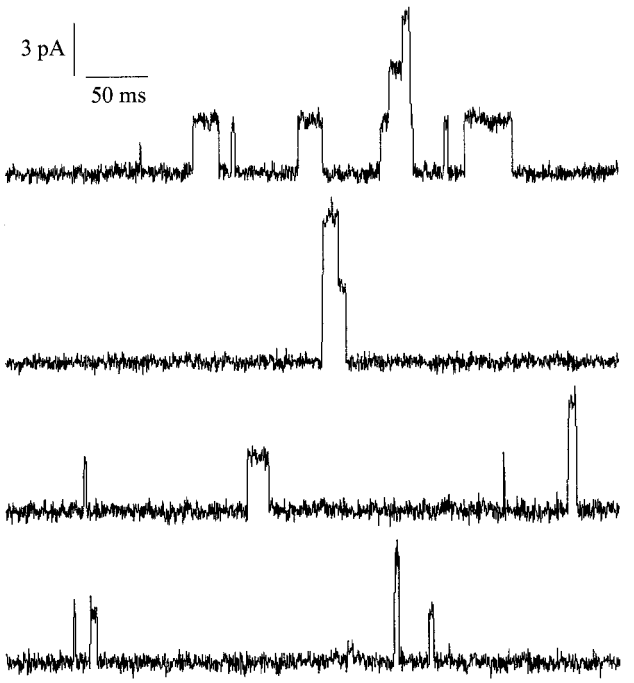

FIGURE 2 Cell-attached single channel recordings from acetylcholine receptors in chick myotubes in the presence of 0.2 μM acetylcholine. Note the apparent simultaneous openings and closures of multiple channels, suggestive of channel cooperativity. Representative traces were taken from separate patches, all with an applied pipette voltage of +20 mV. Small differences in the amplitude of single channel currents between traces were likely due to variation in membrane potential across cells.

where only one active acetylcholine receptor was seen under identical recording conditions (Liu and Madsen, 1996). As expected (Chang and Kurokawa, 1995), the longer the

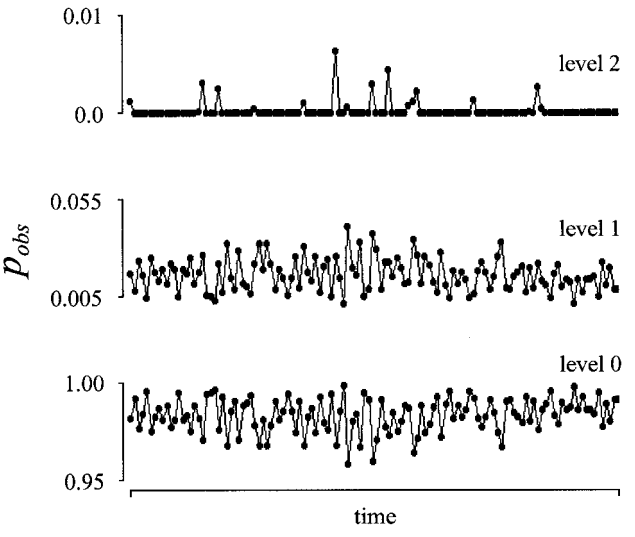

FIGURE 3 Observed probability of occupancy of different conductance levels in consecutive 5-s segments from a 10-min portion of a recording containing two active channels (data set 3, Table 2).

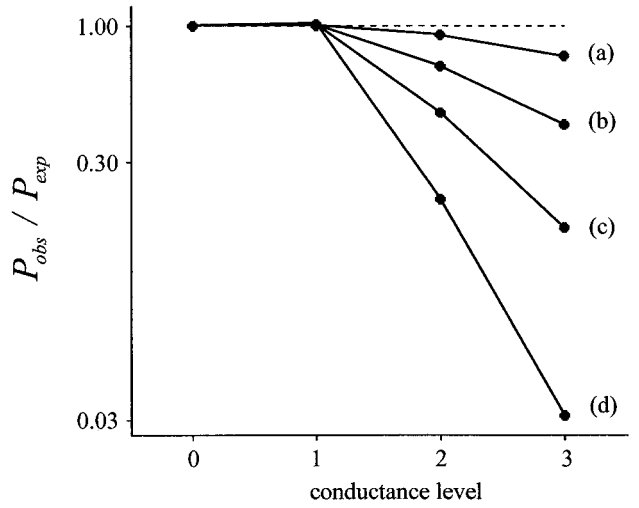

FIGURE 4 Ratio of observed to expected steady state probabilities for a simulated three-channel system, with expected probabilities derived from the binomial distribution (assuming three independent and nonidentical active channels) in four separate cases (*a–d*). The steady-state open probabilities $p_o^{(1)}$, $p_o^{(2)}$, and $p_o^{(3)}$ of channels 1–3 are, respectively, (a) 0.044, 0.051, 0.019; (b) 0.020, 0.078, 0.015; (c) 0.091, 0.008, 0.013; and (d) 0.102, 0.006, 0.003. The probability of the patch not conducting is 0.89 in all cases. The dashed line depicts the expected ratio under binomial assumptions where $p_o^{(1)} = p_o^{(2)} = p_o^{(3)} = 0.0381$. Note the logarithmic ordinate scale.

simulation, the more likely would it be that concurrent openings of all channels were seen. In the case of a patch with two channels, any experimental record longer than 1–2 min would likely ensure double openings are visually observed. For patches with three channels, at least 10 min would be required to have better than an 80% chance of observing concurrent openings of all channels. However, with four channels, even a recording length of 20 min was unlikely to show four concurrent openings, and thus under these circumstances the true value of N would probably be underestimated. From the same simulation studies, for a given length of simulated data, it was also possible to compute the probability of observing at least one instance of two, but not more, concurrent openings when there may have been up to four active channels present (see Fig. 6 for the case of no more than four active channels with duration of 11 min corresponding to the length of the experimental record for data set 2). These simulations suggest that if the experimental data are >11 min long and no more than two concurrent openings are seen (as for data sets 2 and 3), the patch most likely contains only two active channels.

In summary, the frequent observation of rapid transitions across multiple conductance levels and the apparent lack of fit of the steady-state probabilities to a binomial distribution is suggestive of cooperativity between channels, but not necessarily conclusive. Even though the steady-state nature of channel activity was established before examination of conformity with the binomial distribution, the possibility that the observed differences were due to factors other than cooperativity had to be considered. It could have been that channels were not identical and/or the number of channels in the patch was underestimated. The simulation studies shown in Figs. 5 and 6 suggest that the latter was likely for patches with more than two active channels (data sets 1 and 4) but most unlikely for data sets 2 and 3. Regarding the question of channels being identical, the analytical results presented in Fig. 4 suggest differing open probabilities between channels is also unlikely, and analysis of comparable records containing only singly active channels (Liu and Madsen, 1996) indicated, on the basis of a low coeffi-

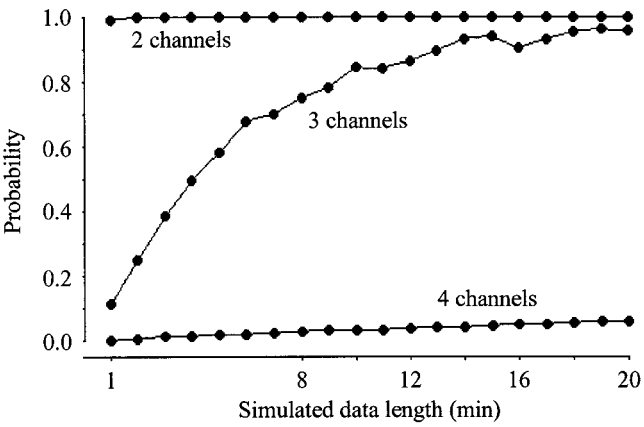

FIGURE 5 Probability of observing at least one occurrence of all channels being concurrently open as a function of simulated data length, in patches with two, three, and four independent and identical channels. Data were generated by sampling the open and closed time density functions obtained from patches with a single active nicotinic receptor present (Liu and Madsen, 1996).

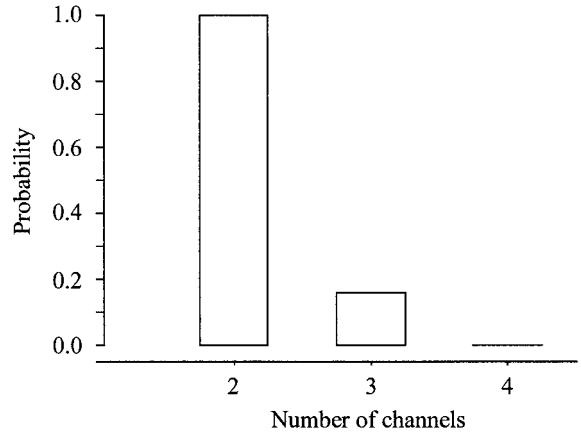

FIGURE 6 Probability of observing at least one instance of two, but not more, concurrent openings in patches with two, three, or four active, independent, and identical channels in a simulated record of 11 min duration. Data were generated by sampling the open and closed time density functions obtained from patches with a single active nicotinic receptor present (Liu and Madsen, 1996).

cient of variation of channel parameters across patches, a homogeneous group of acetylcholine receptors of the fetal type.

Nevertheless, nonidentical kinetics of individual channels due to, for example, subtle differences in posttranslational modifications to individual receptors or differing localized environments could still be possible in the case of multiple channels (such an example was considered by Dabrowski and McDonald, 1992). Accordingly, the two data sets with no more than two concurrent openings (data sets 2 and 3 in Table 2) were studied further to examine the possibility that apparent cooperativity between channels was due to kinetically nonidentical receptors. Channels were assumed to be nonidentical and cooperative, allowing the set of experimental conditional density functions for each channel to be computed with use of optimization techniques on the expanded Markov process. This approach inherently included the other three cases of identical/cooperative, identical/independent, and nonidentical/independent channels. These densities are depicted in Fig. 7 for data set 3, with comparable results being obtained for data set 2. The near-overlapping open time densities within each of Fig. 7, *a* and *b*, suggest that the channels are essentially identical in respect to open times. The shift in location of the densities in Fig. 7 *a* compared with 7 *b* may suggest some cooperativity between channels on closing processes. Fig. 7, *c* and *d*, concerning closed time densities, provide complementary support for channel identity but suggest much larger cooperativity in channel opening processes. The obvious question that arises from these findings is whether the model optimization, under conditions of finite data sets, has the ability to detect nonindependence and nonidenticality. This was tested by simulation of the four possible two-channel scenarios, namely 1) nonidentical and cooperative, 2) identical and cooperative, 3) nonidentical and independent, and 4) identical and independent.

The four sets of results on simulated recordings (each of 10 min duration) using the single channel parameters in Table 1 are shown in Figs. 8–11 (*solid lines* correspond to the conditional densities of either channel obtained from simulation studies, while the *dashed curves* are the analytical solutions). Fig. 8, for example, depicts the case where the channels are nonidentical and cooperative. The closed time densities of either channel (channel 1 and 2) condi-

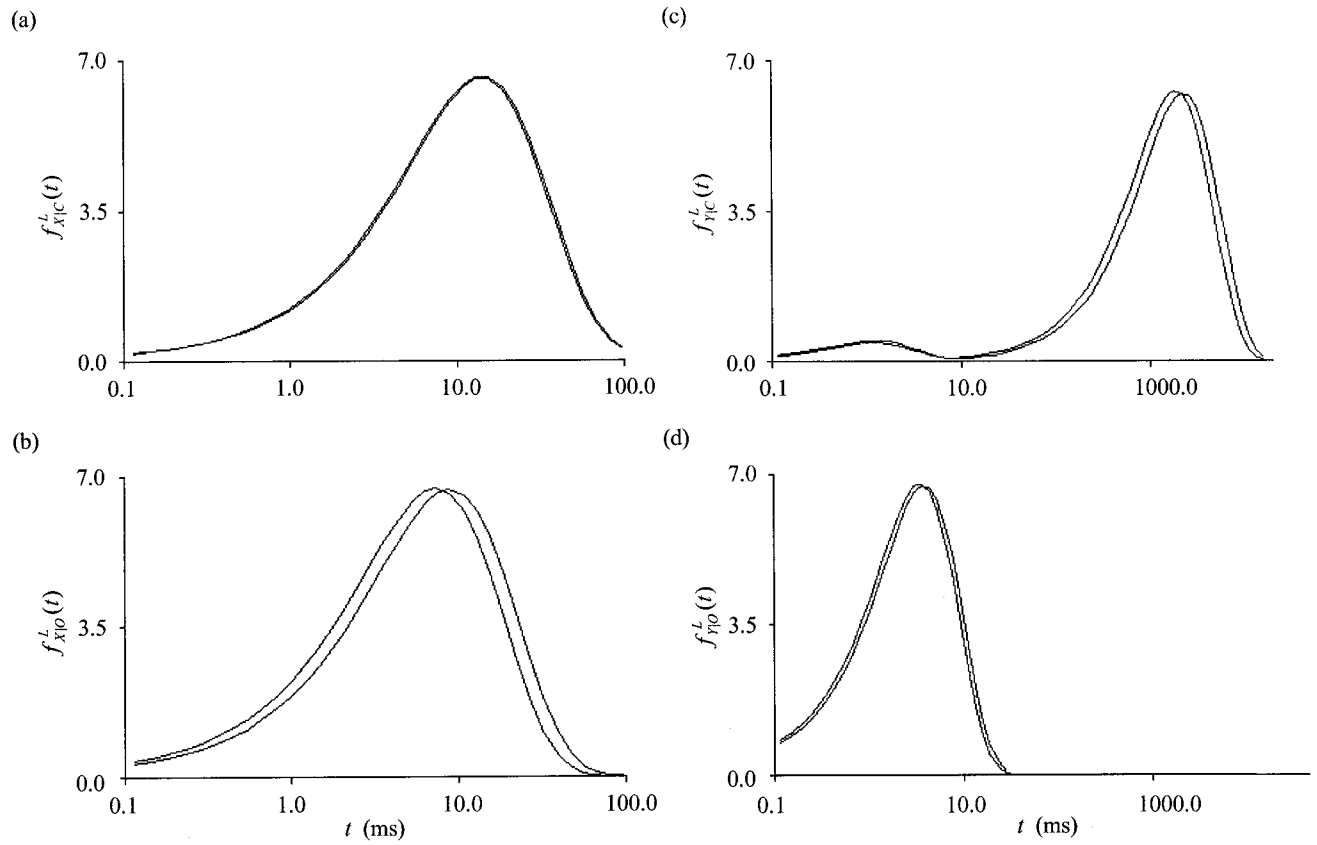

FIGURE 7 Log-binned conditional density functions for individual channels of data set 3 (patch clamp recording with two active acetylcholine receptors). Curves are plotted for $t > 100$ μs corresponding to the approximate dead time of the recording system. (*a*) Open time conditional density functions of channels 1 and 2 given the neighboring channel is closed. (*b*) Open time conditional density functions of the same channels given the neighboring channel is open. (*c*) Closed time conditional density functions of the same channels given the neighboring channel is closed. (*d*) Closed time conditional density functions of the same channels given the neighboring channel is open.

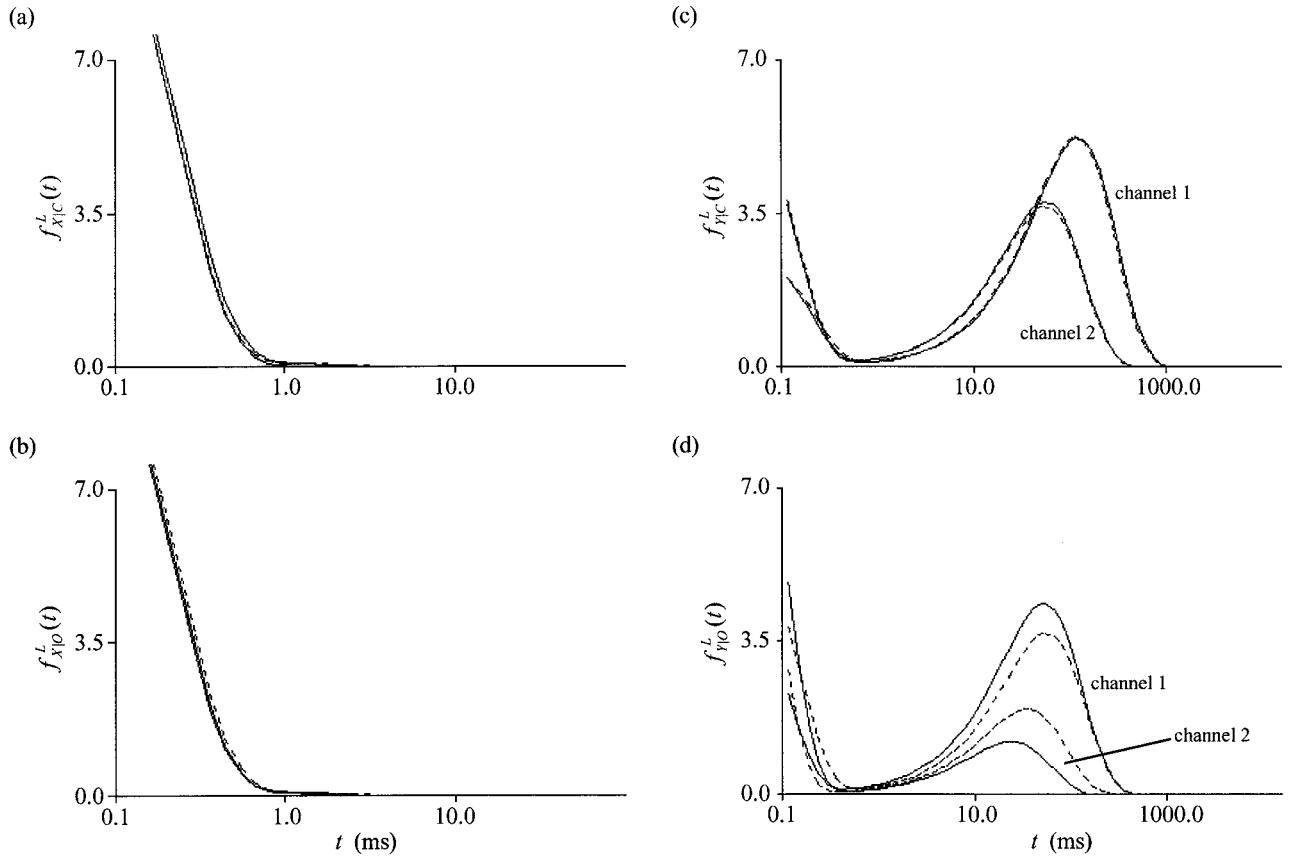

FIGURE 8 Log-binned conditional density functions for individual channels, obtained analytically (*dashed lines*) and by optimization (*solid lines*), from simulated data (Table 1) in the case of two cooperative and nonidentical channels. Curves are plotted for $t > 100$ μs. (*a*) Open time conditional density functions of channels 1 and 2 given the neighboring channel is closed. (*b*) Open time conditional density functions of the same channels given the neighbodring channel is open. (*c*) Closed time conditional density functions of the same channels given the neighboring channel is closed. (*d*) Closed time conditional density functions of the same channels given the neighboring channel is open. Note that the two analytical results in each of (*a*) and (*b*) are equivalent.

tional on its neighbor being closed are shown in Fig. 8 *c*. Because the channels are not kinetically identical (due to differing opening transition rates) the analytical solutions of the conditional densities for either channel (*dashed curves*) are not equivalent. The corresponding results obtained from simulated data by optimization (*solid curves*) closely follow the expected analytical solutions. Similarly, the conditional closed time densities of either channel given its neighbor is open (Fig. 8 *d*) are not equivalent, again secondary to differing channel kinetics. Discrepancies between the optimized and expected analytical results are a consequence of sampling and estimation errors. The increase in opening transition rates in one channel when the other opens (the channels being cooperative) would change the closed time density of the first channel. Indeed, comparison of the curves from Fig. 8 *d* to those from Fig. 8 *c* show such a change in the corresponding conditional closed time densities such that the mean closed time of either channel is less when the neighboring channel is open compared to when it is closed. The curves in Fig. 8, *a* and *b* depict the conditional open time densities for either channel obtained analytically and from the simulated data. Because the weights of these biexponential densities are a function of the opening transition rates (individual channels modeled as in Scheme 1), one would expect the analytical solutions for either channel in Fig. 8, *a* and *b* to differ (the channels being nonidentical), as well as a shift of the corresponding curves between Fig. 8, *a* and *b* (the channels being cooperative). However, these differences were not observed because the ratios β_1'/β_1 and β_2'/β_2 for both channels were equal. Hence, the set of open time conditional densities ($f^{(1)}_{X|C}(t)$, $f^{(1)}_{X|O}(t)$, $f^{(2)}_{X|C}(t)$, $f^{(2)}_{X|O}(t)$) were all equivalent. Note that the results obtained by optimization were in agreement with those obtained analytically.

Figs. 9–11 depict the results obtained in the cases where the channels are cooperative/identical, independent/nonidentical, and independent/identical, respectively. In each case, the presence or absence of cooperativity between the channels and whether they were identical or not was usually interpreted correctly from the conditional density functions, although sampling and estimation errors were compounding

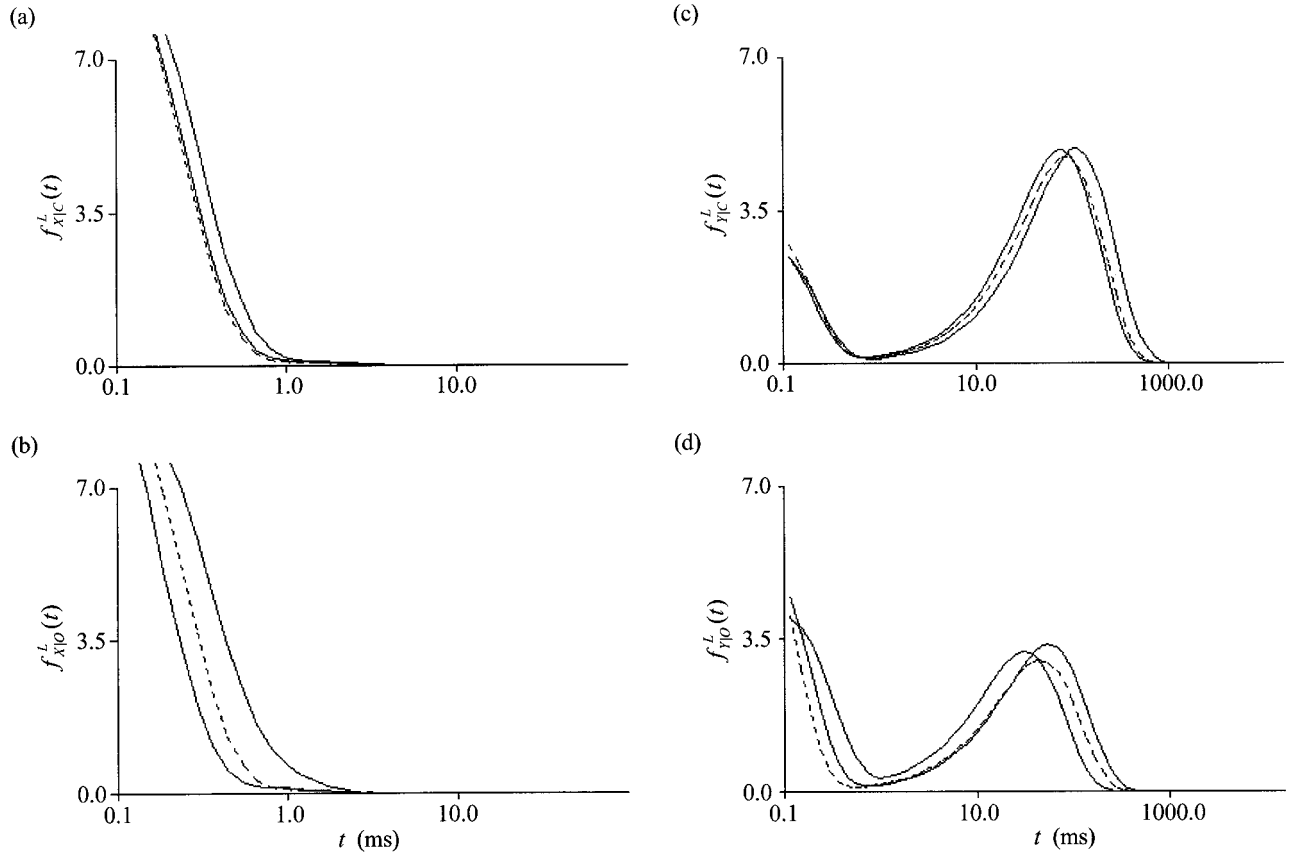

FIGURE 9 As in Fig. 8 but for the case of two cooperative and identical channels. Note that the two analytical results in (*a*)–(*d*) are equivalent.

factors in some instances. It was found that optimizing the transition parameters of the expanded Markov process for the simulated and experimental data to obtain the conditional densities was very computationally intensive. Examination of a broad matrix of different parameter values, for the simulated cases, confirmed the solution space had many local minima. However, solutions with a good fit to the steady-state probabilities and densities of the six sojourn types gave remarkably similar conditional density functions.

Inasmuch as the two concurrently active channels in data sets 2 and 3 could be considered kinetically identical, $K_C(t)$ and $K_O(t)$, comparing conditional sojourn time density functions, could be calculated (see Fig. 12). The shape of $K_C(t)$ in each case was approximately similar, with a peak much greater than unity at $t \cong 10$–15 ms, followed by a monotonic decrease toward unity for large t. Likewise, the shape of $K_O(t)$ for both data sets was similar, but in this case there was very little difference from the independence prediction (i.e., $K_O(t) \cong 1.0$ for all t).

DISCUSSION

There appears to be good evidence for channel (or co-channel) independence in several systems (Ludewig et al., 1997); however, in many cases independence is simply assumed because it represents a starting point for understanding macroscopic ion channel behavior. Regarding the nicotinic receptor, an early examination of this question was made by Neher et al. (1978) who concluded that steady-state probabilities in single channel recordings of denervated rat diaphragm muscles were consistent with the Poisson distribution, suggesting channel independence. More recently, Lui and Dilger (1993) found that nicotinic receptors from BC3H-1 cells showed no measurable interaction between channels using a one-dimensional Ising model when the variance of the recording was plotted as a function of open probability (p_o) at different concentrations of acetylcholine. However, cooperative effects between nicotinic receptors in *Torpedo* electric tissue were reported by Schindler et al. (1984). They obtained evidence for positive cooperativity between channels, with gating of pairs of receptors so strongly coupled that the channels seemed to open and close synchronously. This behavior was independent of the covalently linked disulfide bridge connecting receptor monomers in *Torpedo* membranes. In addition, they noted higher-order interaction effects (i.e., association among two, four, and six individual channels). In a study by Yeramian et al. (1986) using a variable time window to scan experimental

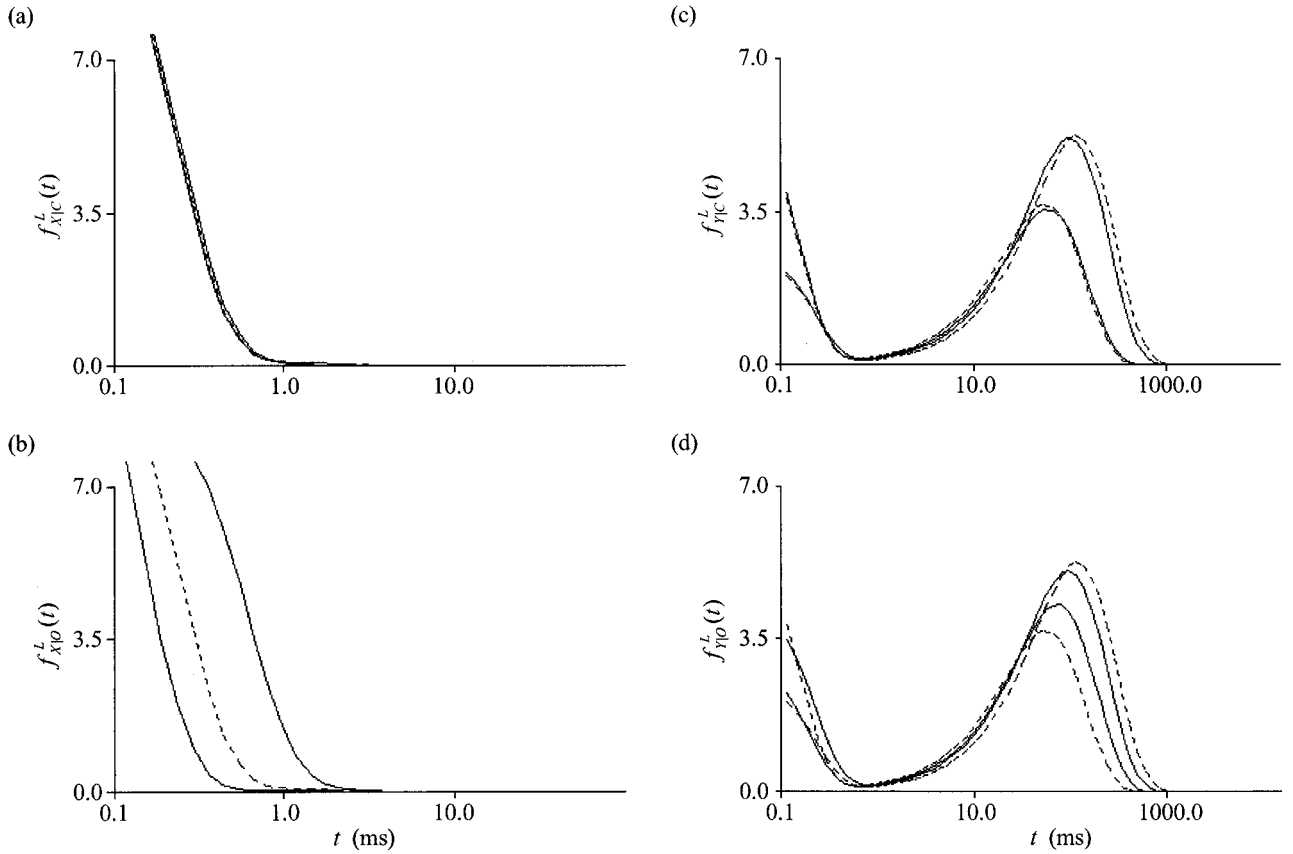

FIGURE 10 As in Fig. 8 but for the case of two independent and nonidentical channels. Note that the two analytical results in (*a*) and (*b*) are equivalent.

recordings, it was found that multiconductance level transitions occurred more often than predicted under an independence assumption. They concluded that pairs of receptors interacted in a positively cooperative manner.

Comparison of steady-state probabilities of occupancy of different conductance levels in patch clamp recordings with predictions from the binomial distribution offers a simple test for possible interaction between channels. Although this approach does not require knowledge of the detailed chemical reaction scheme for channel activation, underlying assumptions include correct estimation of the number of channels in the system, the channels being independent and identical, and constant open probability throughout the length of the recording (i.e., steady-state conditions). In the present study of chick skeletal muscle nicotinic receptors where the numbers of active channels in data sets 2 and 3 were confidently estimated as equal to the maximum number of concurrent openings, the observed probabilities for multiple openings were greater than those predicted by the binomial distribution. These cell-attached recordings were at steady state (Fig. 3), and channels in the patch were identical, as evidenced by the close similarity of conditional density functions shown in each of Fig. 7, *a–d*. Receptor desensitization and "buzz modes" found in some channels (for example, McManus and Magleby, 1988) are phenomena that could cause a change in channel open probability throughout a recording, but neither was evident in the four data sets studied. Hence, a possible explanation for the consistent discrepancy in the ratio P_{obs}/P_{exp} seen in Table 2 is that interaction between channels occurs such that gating behavior in one channel is influenced by activity in a neighbor. For example, a system in which the opening of one channel increases the probability of other channels opening, or alternatively the closure of one channel decreases the probability of other channels closing, would result in the observed distributions of the ratio P_{obs}/P_{exp}. This suggests that nicotinic receptors interact in a positively cooperative manner, but the binomial analysis provides no further information regarding the underlying mechanism of interaction.

Some additional evidence for cooperativity in these data sets was obtained from an analysis of multichannel data used by Manivannan et al. (1996). In this approach, the kinetic behavior of each channel is described by a continuous-time Markov process and varying numbers of channels are assumed to cluster together, the consequence of aggregation being that all constituents then gate as a single unit. Hence, this model represents a case of extreme positive

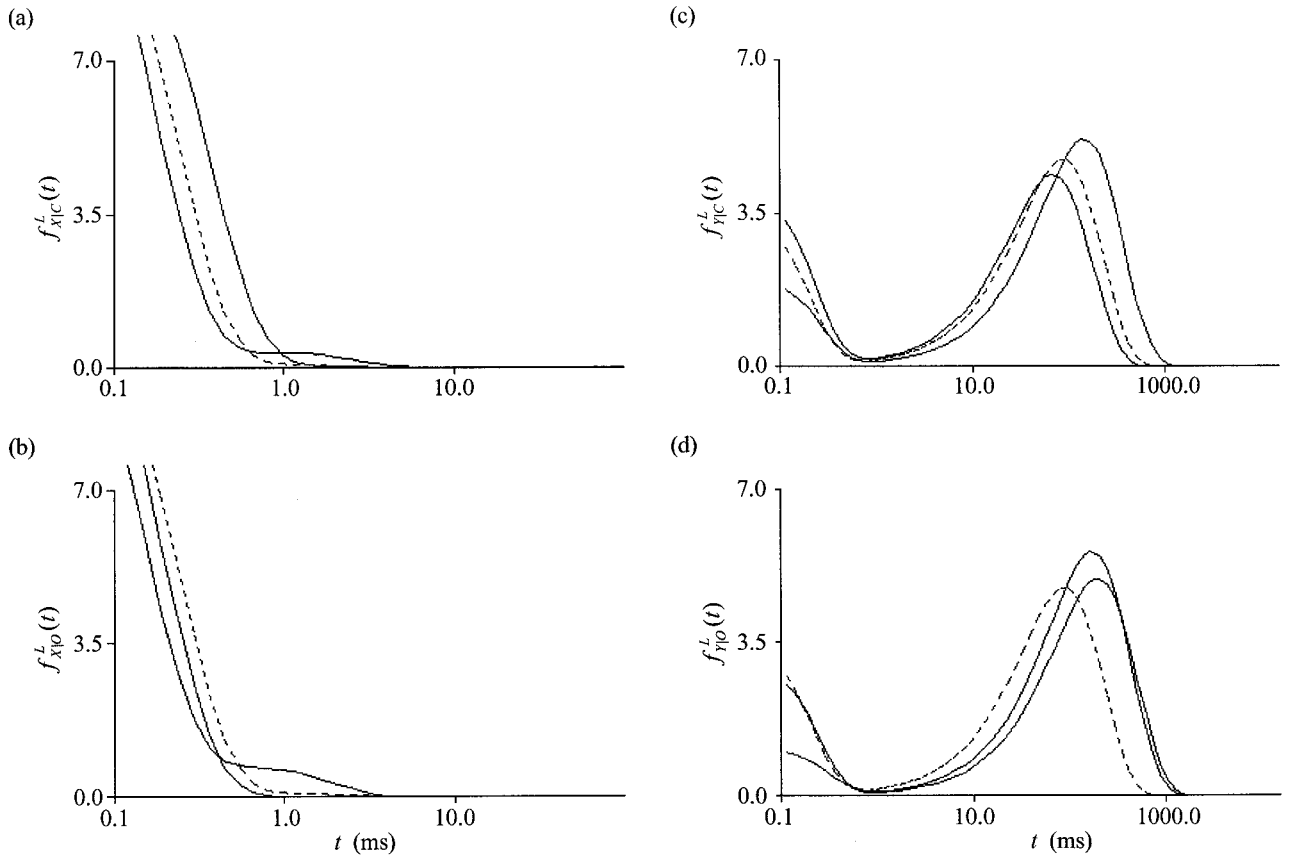

FIGURE 11 As in Fig. 8 but for the case of two independent and identical channels. Note that the two analytical results in (*a*)–(*d*) are equivalent.

cooperativity, where the coupling of channel gating is not merely enhanced compared to independent behavior, but is necessary and instantaneous. Although the model can describe some features of the distributions found in the present study (results not shown), the theory underlying the analysis assumes a simple two-state Markov model for the single channel kinetics, and the effect of considering a larger state space more representative of nicotinic receptor behavior (i.e., Scheme 1), is unknown.

The conditional density functions computed by optimization under the most general assumption of possible channel cooperativity and nonidenticality (Fig. 7 for data set 3) show minimal differences between the two channels in each of the conditional densities, suggesting identical kinetics. However, although the open time densities conditional on the neighboring channel being closed or open are similar (within sampling and estimation limitations), this is not the case for the closed time conditional densities where the mean closed time of a channel is much larger when its neighbor is closed than when it is open. This time shift in the densities $f_{Y|C}(t)$ and $f_{Y|O}(t)$ suggests cooperativity between the channels on opening processes, whereas the much smaller differences between $f_{X|C}(t)$ and $f_{X|O}(t)$ suggest closing processes between channels are relatively independent.

The above findings were illustrated graphically using functions $K_C(t)$ and $K_O(t)$ (Fig. 12). These relate conditional sojourn time density functions and are of the general form $1 + \Sigma_{i=1}^{n} w_i \exp(-\lambda_i t)$, where n is related to the number of states in the underlying single channel kinetic scheme, and all w_i are equal to zero if the channels are independent (Keleshian et al., 1994). In the two data sets with $N = 2$ (sets 2 and 3), both $K_C(t)$ and $K_O(t)$ decayed as expected to 1.0 for large t, but $K_C(t)$ for shorter times (i.e., $t < 100$ ms) was markedly greater than unity (Fig. 12), suggesting cooperativity between channels. Given the small increase in the ratio of observed to expected steady-state probabilities for multiple openings in Table 2 (suggesting noncompliance with binomial statistics), the current approach based on conditional density functions may be, in some cases, a more sensitive indicator of cooperativity than the binomial distribution. The inability of binomial analysis to detect cooperativity in all situations has been reported by others (Uteshev, 1993). Because $K_C(t)$ (which compares conditional closed time density functions) is greater than unity, and given the results in Table 2 suggest positive cooperativity, the present findings are compatible with a model where the opening of one channel increases the likelihood that the other will open through increased binding of acetylcholine (changes in k_{on}

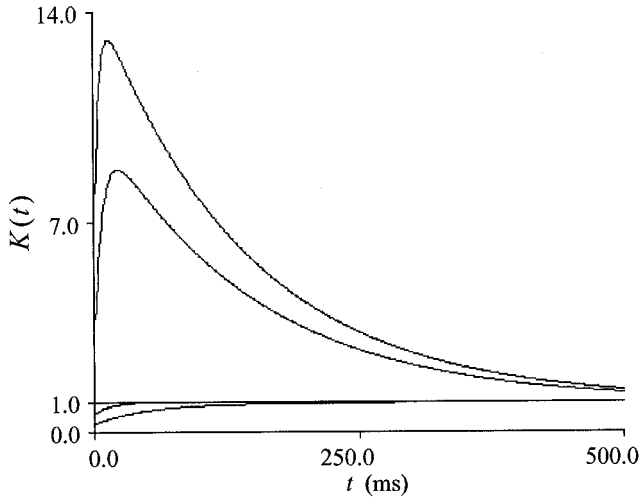

FIGURE 12 $K_C(t)$ and $K_O(t)$, collectively denoted as $K(t)$, obtained from two data sets where a maximum of two concurrent openings was observed (data sets 2 and 3). The line at $K(t) = 1.0$ denotes the expected result if the conditional density functions related to the closed times ($f_{Y|C}(t)$ and $f_{Y|O}(t)$) and open times ($f_{X|C}(t)$ and $f_{X|O}(t)$) were identical (i.e., channels independent). The traces above $K(t) = 1.0$ correspond to $K_C(t)$ for data sets 3 and 2 (from *top* to *bottom*, respectively) while the ones below $K(t) = 1.0$ are $K_O(t)$ for the same two data sets (plot for data set 3 being above the plot of data set 2).

and/or k_{off}), increased channel opening rates (changes in β_1 and β_2), or both of these processes.

Changes in $K_O(t)$ were very small compared with those for $K_C(t)$ under comparable conditions and were within noise and estimation errors as shown by the simulation studies. It is therefore unlikely that they provide evidence for cooperative effects between channels on the closing transition rates α_1 and α_2. However, inspection of the function for $K_O(t)$ in closed form,

$$K_O(t) = 1 + \left[\frac{(\alpha_2'(w_1 - w_1') + \alpha_1'(w_1' - w_1))}{(\alpha_2' w_1' - \alpha_1'(1 - w_1'))}\right]\exp(-At),$$

where

$$w_1 = k_{on}\beta_2/(k_{on}\beta_2 + 2k_{off}\beta_1),$$

$$w_1' = k_{on}'\beta_2'/(k_{on}'\beta_2' + 2k_{off}'\beta_1'),$$

and

$$A = \alpha_1'\alpha_2'/(\alpha_2' w_1' + \alpha_1'(1 - w_1'))$$

shows that it is theoretically possible in some special cases to observe unity values of $K_O(t)$ even though closing processes are nonindependent, for example when $\beta_1\beta_2' = \beta_1'\beta_2$.

Summarizing the kinetic analysis in the two-channel system, comparison of the interaction function $K_C(t)$ suggests that the open states of one channel might increase binding affinity of acetylcholine, opening rate, or both these properties in the other channel. This mechanistic interpretation compares with the study by Iwasa et al. (1986) where it was concluded that (negative) cooperative interactions between batrachotoxin-modified Na^+ channels were due to effects on channel opening rate. As was the case in our study, no evidence was found for interactional effects on channel closing transition rates. Possible physical mechanisms for the cooperative effect could include a direct protein-protein interaction whereby opening of one channel causes binding sites in the neighboring closed channel to be more accessible (perhaps sterically compatible or electrically attractive) to the positively charged acetylcholine molecule. Consistent with the present finding of interaction between (at least) two channels, it is interesting that structural evidence of transient dimer formation in response to acetylcholine release has been obtained using rapid-freezing and cryofracture techniques in the electric organ of *Torpedo* (Dunant et al., 1989). Similarly, coupled gating in ryanodine receptors has been explained by dimer formation (Marx et al., 1998). Draber et al. (1993) studied the behavior of K^+ channels in *Chara corallina* and suggested cooperative interactions in this preparation may be due to mechanical interaction between channels resulting from gating conformational changes. Alternatively, interacting channels may not be in physical contact, but altered surface charge resulting from conformational changes in one channel may have an effect on a neighbor, depending on proximity and surrounding ions. Interaction resulting from such fluxes has been studied theoretically by Berry and Edmunds (1993) using a multipore channel model. Furthermore, although not strictly compatible with the present analysis, where it has been assumed that gating of one channel results in an instantaneous change of transition rates in its neighbor, diffusion of permeant or nonpermeant ions in the microenvironment surrounding channels could, if restricted, cause an accumulation of charge near the outer vestibule that might attract acetylcholine and thus increase effective local concentration. Similar intracellular submicroscopic Ca^{2+} diffusion has been reported to cause inhibitory coupling between individual Ca^{2+} channels (Imredy and Yue, 1992). Finally, a number of theoretical approaches separate from that presented here (e.g., Morier and Sauvé (1994); Chung and Kennedy (1996); Klein et al. (1997); Blunck et al. (1998)), have now been developed to assist assessment and interpretation of cooperative ion channel behavior.

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