DISCUSSION

Session Chairman: Harold Lecar *Scribes*: Thomas N. Earnest and Sarah S. Garber

LECAR: In the prototypical scheme for AChR kinetics, an empty receptor becomes singly liganded, remaining in a closed state, then becomes doubly liganded and undergoes a conformational transition which leads to an open state. One consequence of this scheme is that whatever else becomes complicated, the histogram of open state lifetimes obeys a single exponential. All the channels that close are closing by a Poisson process.

In this paper and in work done in tissue culture cells, there is evidence for histograms of open state lifetimes that do not seem to obey a single exponential. This is not just a question of multiple pathways from the open state, but rather two exponentials that would mean two distinct open states. Two pathways from an open state would merely give a single exponential with an altered rate constant. So there is a disparity in single channel work at the present; maybe it has to do with the statistical data processing or maybe alternative open states of the AChR molecule are expressed in some environments. It is striking that the bilayer kinetics do resemble the kinetics obtained from tissue culture cells.

MONTAL: In 1980, when we began to get longer single channel recordings, we found that distribution of open times could not be fit by a single exponential, and we were quite concerned. We later found out that this was also observed in tissue cultured muscle cells and in native muscle preparations. David Tank has reported that this occurs in patch clamped liposomes, and Alfred Maelicke has also found two open states of AChR in reconstituted systems.

LEIBOWITZ: It is clear that many people are seeing multiple components of the open-time data. Comparison of many different preparations does not tell us more about the function of the neuromuscular junction (NMJ). This is not the important consideration. It is the physiological function of the multiple conduction states that we want to understand.

MAZET: Given the single channel activity, the macroscopic current should be smoothly decreasing. Does the large fluctuation of the macroscopic current that you see reflect the same working mode of the receptor as the single channel activity or does it reflect the cooperative activity observed by Hans Georg Schindler?

MONTAL: The transient overshoot in the macroscopic response can be accounted for quantitatively by desensitization. Fredkin has simulated the macroscopic response by addition of single channel records.

TANK: There is evidence that the phosphorylation state of the *Torpedo* AChR changes during development. Have you compared the single channel properties of the receptor isolated from *Torpedo* neonates with those of the adult?

MONTAL: No. But we intend to test whether covalent modification of the protein could account for changes in the conductance states. It is known that the receptor can be phosphorylated or methylated. These covalent changes could account for changes during maturation of the receptor.

SACHS: The presence of two conductance states does complicate the kinetic model, but they are found at end plates and they show up in the isolated receptor as well. There is a suggestion of a fast component to the open time decay in Fig. 4 *b* of Leibowitz and Dionne. Whether or not the fast component is seen may depend on the value of the time constant of the particular channel. If the time constant is 200 μs or more, it should be seen; if the time constant is 100 μs, it could be lost in the noise. It may be that the lack of two open times is an artifact, rather than an experimental observation.

LECAR: An obvious interpretation of the other open state is the existence of a mono-liganded open state (AR*). This interpretation may be inconsistent with the data, but it is a simple, satisfying interpretation that provides a tangible explanation for two time constants.

FINKELSTEIN: The picture you have of the bursting behavior involves movement of the channel from AAR to AAR*. How does one preclude that bursting is not due to the AAR* state going into another closed state, distinct from the inactivated state, and that this is what you are seeing as flickering of the channel?

MONTAL: Nothing precludes that possibility. We tried to be more simplistic by using a singly liganded state rather then a doubly liganded state. But there is an additional state of desensitization. The desensitization acts like a sink to which the receptor can go and recover into the active state. This was proposed by Sakmann et al. a few years ago (Sakmann, E., J. Patlak, and E. Neher. 1980. *Nature* (*Lond.*). 286:71–73), and it can account quantitatively for the bursting.

DIONNE: We have used the simplest model to fit our data

$$2A + R \rightleftharpoons AR + A \rightleftharpoons A_2R \rightleftharpoons A_2R^*$$

at the lower limit of agonist concentration. Of course more complicated models can be used, introducing more closed and open states. This introduces parameters in the curve-fitting schemes but does not provide more information, since these new parameters are freely adjustable. Therefore one uses the simplest model to fit the data, and tries to make inferences from this.

Leibowitz and I have also seen open time distributions with two components, but not always. Have you manipulated any conditions such as lipid composition, and observed changes in the distribution of open time durations?

MONTAL: No. This is one of our next steps.

GARDNER: Concerning the two populations of open states, you have used the principle of conditional probabilities and find a correlation between the probability of long openings and the probability of short openings. You use this to argue against two populations of channels. Is it possible that some other parameter may vary, such as the concentration of ACh, and that could be driving two independent populations of channels?

MONTAL: In principle there could be some other parameter. But we have applied the conditional probability argument over two orders of magnitude of agonist concentrations, and the situation still prevails.

EISENMAN: Does the receptor stick out far enough from the membrane so that it is insensitive to the surface charge of the lipids, or is it possible the channel properties could be modified by the surface charge?

LABARCA: We don't have a precise answer to your question. The receptor does stick out of the *cis*-side of the membrane (against-binding side) by some 55Å and by ~15Å from the opposite side. The channel might be modified by surface charge in the lipid or more probably, by charge residues in the protein itself. In our hands the apparent K_d of saturation is probably affected by the concentration of calcium ions. There is also evidence that calcium modifies the receptor properties at end plates.

MONTAL: There are technical problems in using pure lipid compositions for reconstitution. Thus far, mixed lipid compositions must be used for optimal reconstitution activity.

DONOVAN: There are two classes of models we have discussed. One is derived from the physiology, where we talk of ligand binding and the subsequent opening of the channel; the other is derived from formal kinetic schemes, in which we try to fit parameters from electrical measurements. It is important not to blur the two. For example, using ligand binding rates, you stated that the double exponential closed times cannot be fit by a three state model; but if approached strictly from a formal kinetic model, a double exponential can easily be fit using a three state model. Similarly, the k_{-2} found by Liebowitz and Dionne may not necessarily be a ligand unbinding step.

DEFELICE: You can think of the flickering in one of two ways: returning to the closed state or going to a special closed state, as comes up often in voltage dependent channels. It is not clear what one means when one speaks of the simplest interpretation for fitting the data. Are there experiments that one could do which could distinguish between a closed-open-closed and a closed-closed-open model?

DIONNE: The formalism demands three states. We assume the initial binding states to be at equilibrium; in the low concentration limit this gives a C-C-O model which makes only slightly different predictions from a C-O-C model. There are some experiments that could distinguish between these schemes but they haven't been done.

MANNELLA: There is evidence that the receptor can exist in dimer form. Is there any correlation between the number of lifetimes that one sees in different systems and the concentration of receptors that you would expect there to be in the membrane, so that the difference in conductance might correlate with different aggregation states of the single channel that you're looking at?

MONTAL: We have carried out flux measurements on reconstituted vesicles in which we have incorporated exclusively monomers or exclusively dimers, and we see no differences (Anholt, R., J. Lindstrom, and M. Montal. 1980. *Eur. J. Biochem.* 109:481–487). We have not pursued the studies at the level of the single channel, but Hans Georg Schindler has done these measurements and found some differences between monomers and dimers.

MOCZYDLOWSKI: You mentioned that you've made measurements over several orders of magnitude of agonist concentration. Do you expect that the singly liganded closed state can open and could this be an explanation of the two-exponential open state distribution? Is there a change in the amplitude of the exponentials with a change in agonist concentration? What about the distribution of closed state lifetimes?

MONTAL: We are studying these questions systematically. We have not found any amplitude changes so far with a change in agonist concentration; the ratio of the amplitudes of the fast and slow components seems to be constant.