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# DISCUSSION

*Session Chairman*: Harold Lecar *Scribes*: Malcolm Hunter and John W. Hanrahan

KOSOWER: Short and long open states might arise, whatever the structural model, from the nature of the ACh receptor. It could be $\alpha, \beta$; $\alpha, \gamma, \delta$; or $\beta$ and $\gamma$ might be switched — see my paper at this meeting. This might apply to any structural model. If you put the ACh in one way, a particular type of opening occurs, whereas if you put it in another way, you may see a similar, but not exactly the same, type of opening. If the binding site is in the channel, this might account for the two different types of openings.

PATLAK: There is a certain amount of confusion about names for the various groupings of openings and closings. Steinbach has a very sensible set of terminologies in this paper that might be universally adopted. At low concentrations of agonist, one usually sees openings that are interrupted by brief closures such as the "Nachschläge" phenomenon, i.e., openings associated with one another. They have been referred to in the literature as "bursts. " The original desensitization phenomena, referred to as "paroxysms" by Montal and "bursts" by Sakmann et al. (Sakmann, E., J. Patlak, and E. Neher. 1980. *Nature* (*Lond.* ). 286:71–73) is a much longer grouping of channel openings. Joe Henry Steinbach has called these "groups. " There is an even longer process, an association of these groups themselves; Steinbach has followed the Sakmann et al. terminology by calling these "clusters. "

My question has to do with the variability of these channels with age of the cultured cells. Do you think the channels that are present on day 10 are are still present on day 18, and that these are subjected to some time-dependent change? Or are the cells synthesizing new but different channels?

STEINBACH: The half-life of these receptors is ~12 h, so it is extremely unlikely that the channels present on day 10 are still present on day 18.

DANI: There were often five exponentials in the closed-time distributions that you showed. Is it possible that some of these components are arising because channels are being lost in a way that is different from desensitization, and might be referred to as "run-down" in other preparations. I notice that I lose channels, and it isn't some artifact of the patch-clamp technique such as sealed vesicles.

STEINBACH: Particularly at high concentrations, if you plot the opening frequency over intervals through the record, the regression line is flat, the slope is not significantly different from zero. With regard to long term stationarity, I don't know how to test exactly how stationary these records are because they are too short to detect very slow changes.

HORN: The complexity of the kinetics could mean that each channel has very complex kinetics, or that there is a heterogeneous population of channels having the same conductance. Is it possible to detect channel heterogeneity? Do you have any evidence that this is a homogeneous population other than their having the same conductance?

STEINBACH: First, we think that although brief and long duration open states are present, they both can be produced by a single receptor. The close temporal relationship of brief and long events at high agonist concentration indicates that it is very likely that either the same receptor channel produces both the brief and long openings or, if two different channels are producing them, there must be strong negative cooperativity to prevent multiple openings with such high duty. For that reason, I think that although the open states are heterogeneous, they arise from a single population of ACh receptors.

We analyzed the mean open time of long duration events in groups that contain brief openings and those that do not contain them, to test the idea that there are two types of channels, one which can make a brief opening and one which cannot. There is no difference there. We counted up the number of groups of openings at different concentrations which had one long opening, two long openings separated by a brief closing and so forth. This tests whether the probability of reopening during a short interval after a closing is constant regardless of the number in the train. In other words, is there one type of channel in terms of the grouping behavior? Plotting the number of groups with $N$ events vs. $N$ on a semi-log plot, the data fall on a straight line, consistent with the idea that grouping behavior is homogenous.

I think that the grouping and the open time behavior are homogeneous. The single channel conductance also shows no dependence on duration. In the older cells, the channels are heterogeneous.

HORN: What about patch-to-patch variation?

STEINBACH: The measured parameters are certainly within twofold or so, a standard error of ± half the mean would be the range. This is similar to the variability that I measured in voltage clamp experiments on neuromuscular junctions.

KOLB: How do you prove in single channel recording that the kinetic process is in equilibrium so that you can apply chemical reaction kinetics?

STEINBACH: I do some tests for stationarity by comparing the event frequencies and mean open times at the beginning and end of a record. There are no significant changes over the length of a 10-min record. On a shorter time scale I do not see characteristic asymmetries; for example, the distributions of durations of closed periods preceding a long opening and following a long opening appear to be time-symmetrical.

KOLB: Regarding the second open state, this might be the case where one ligand binds to the receptor, but do you have any experimental evidence concerning the modulation of the channel from the cytoplasmic side? For example, one of the open states might be generated by the phosphorylation or dephosphorylation of membrane components related to the ACh-activated channel, similar to that found for the Ca-channel in cardiac muscle cells.

STEINBACH: We don't have any evidence regarding that.

KOLB: I want to point out that when one deals with several open states, one should look at modulations of the channel from both the inside and outside.

STEINBACH: We do have indirect evidence that this is not responsible for the two open states because the proportion of brief and long openings is similar in cell attached, inside-out or outside-out patches. We have done a few experiments changing the Ca concentration on the cytoplasmic side and it doesn't seem to affect the relative proportions of brief and long openings. We haven't looked at phosphorylation. We are very interested in the possibility of modification or modulation of receptor function from the cytoplasm.

BENNETT: Why can't the Nachschläge phenomenon be due to a closed state on the other side of the open state (i.e, to the right in the kinetic scheme)? Can miniature post-synaptic currents answer this question? Could one apply what amounts to a $\delta$ function of transmitter, which I suppose one still can't do with a patch or by iontophoresis? Post-synaptic currents at some snake neuromuscular junctions and at the hatchet fish giant synapse show a rapid decay followed by a slow decay. These data can be explained by assuming that the channel goes from a bound-open state to a bound-closed state; the sequence must be reversed before the transmitter can dissociate. Is there still ambiguity regarding whether the Nachschläge are due to a closed state to the left or right of the open state?

STEINBACH: The lack of concentration-dependence of the frequency and time constant of the Nachschläge in our data indicate that the brief closings do not result exclusively from closures back to the left. At least some of them must result from closed states to the right of the open state, and this closed state is not due to block of the channel by agonist.

DILGER: Working on the same cell line, we have developed a technique of adding agonist within ~100 ms to an outside-out patch. We find that several hundred channels are opened in a patch and the number of excitable channels in the patch does not decrease appreciably after 20 min.

LABARCA: You find that the frequencies of the two open states don't change with agonist concentration and the lifetimes also do not seem to change. We have made that observation with purified receptors with several agonists and different concentrations.

SCHNEIDER: I wish to make a general comment about proteolytic enzymes. These may not be completely inactivated by quenching in 2% calf serum. Looking at requirements for synthesis of neural antigens in retinal cultures we have frequently seen lag phases lasting for several days before appearance of the antigens after light trypsinization. We are able to eliminate these lag phases completely by quenching with phenyl methyl sulphonyl fluoride. This lag phase is probably due to adsorption of trypsin onto the cells; the trypsin can remain there for several days despite exhaustive washing. Also, since you have used digestion to enhance the probability of achieving high resistance seals, are the properties altered (with respect to those observed in untreated cells) by this process?

STEINBACH: We always wait at least four days (six to seven half-lives of the receptor) after enzyme treatment, so we believe the receptors we are looking at were not treated. Based on the data that we have, there is no difference between untreated and treated cells.

SACHS: A general comment: If one has agonist in the pipette, one is looking at the steady-state, and desensitization may have already occurred. This would select for channels that do not desensitize, and these might be different from those that determine the macroscopic properties. There is a discrepancy between the number of channels determined by toxin binding (200–1,000/$\mu^2$) vs. those calculated from binomial analysis in patches (3–6 channels).

STEINBACH: We do not see changes in properties of the open receptor channel over the concentration range studied here, so the selection seems to be concentration independent. As Dr. Dilger said, he has found better agreement in binding site and receptor numbers using rapid agonist applications.

MOCZYDLOWSKI: You have provided an estimate of $k_{-2}$, the agonist dissociation rate. How do these values compare with those obtained from binding studies? Could you use an agonist with a long lived residence time as a tool for identifying a certain fluctuation with agonist residency?

STEINBACH: We hope to do this using curariform antagonists as agonists. You would predict from their $K_D$ that residency times would be very long. I do not know of reliable biochemical estimates of the off rate constants for strong agonists.

ROUX: Could you explain how you know that the behavior does not arise from independent processes?

STEINBACH: We demonstrate temporal relationships that could not occur through random association of independent receptor activations. A trials analysis showed that brief openings occurred far too frequently in trials that had long openings given the overall probability of a brief opening occurring in any trial, independent of whether there was a long opening in it. An alternative method is to look for time correlations, to demonstrate temporal relationships between the end of one event and the beginning of others. Looking for such temporal relationships is one of the advantages of the patch-clamp technique.