Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 232–235, January 1985
Neurobiology

Statistical test of linearity of photoreceptor transduction process: *Limulus* passes, others fail

(bump/visual transduction/linear model/single-photon responses)

NORBERTO M. GRZYWACZ AND PETER HILLMAN

The Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem, Israel

Communicated by Floyd Ratliff, August 13, 1984

ABSTRACT **We present the results of a theoretical analysis of a completely general linear chain model for transduction in photoreceptors from which we have derived a statistical test for the intrinsic linearity of the single-photon transduction process. By linearity we mean comprising first-order chemical reactions only. We show results of our own measurements in *Limulus* ventral photoreceptors that pass this linearity test, suggesting that the single-photon transduction in *Limulus* may be a simple chain of first-order biochemical reactions (plus possible diffusional processes). However, we also demonstrate that published data show the existence of strong nonlinearities in the single-photon responses of toad and perhaps also of locust. Such nonlinearities are not difficult to construct from existing biochemical notions (feedback, cooperativity), but all but one [Kramer, L. (1975) *Biophys. Struct. Mech.* 1, 239–257] of the published analytical models of the single-photon process have been linear. The test we have used is the distribution of "areas" (time integrals of conductance changes) of single-photon responses or "bumps." Reasonable molecular linear chain models do not allow distributions very sharply peaked at nonzero values. Such peaked distributions are seen in toad and locust but not in *Limulus*.**

The detailed biochemistry of transduction in photoreceptors is not yet clear. The process underlying the responses of photoreceptors to absorption of single photons is of particular interest, since all photoreceptor responses appear to be made up of these "bumps" modulated by adaptive processes (1). A knowledge of the linearity or nonlinearity of this process would provide an important constraint on possible mechanisms. By linearity, we mean that the output (the response of the photoreceptor) depends on the input (the light intensity) in such a way as to satisfy the classical criterion of linearity of a system, the principle of superposition. A system of first-order chemical reactions and diffusional processes only is linear in this sense. These are the only relevant molecular processes that are linear.

The linearity or nonlinearity of a process can usually be determined directly from the dependence of response amplitude on stimulus strength but, in the case of single-photon processes, this approach is not feasible, as the stimulus strength is fixed at a single photon. Nevertheless, the responses to single photons are variable, reflecting the stochastic nature of the transduction process (2), and we have devised a test for the linearity of the process based on a quantitative examination of this variability. We have constructed and analyzed a model for the process comprising all possible combinations of linear chemical reactions and have confronted the predictions of this model with voltage-clamp observations we have made on *Limulus* ventral photoreceptors and with published data on toad and locust.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

The rigorous analysis of a general linear chain model is long and will be published separately. Here, we justify a specific prediction of this model for bumps by an intuitive argument and by an outline of the analysis. The prediction relates to the distribution of the "areas" of the bumps (the time integrals of the conductance changes).

The argument begins by noting that the probability distribution of the active lifetimes of single isolated enzymatic molecules whose inactivation is a first-order process necessarily has an exponential form, declining from a peak at zero lifetime. In a linear system, the average rate of appearance of the enzymatic product of the single active molecule is constant, so that the distribution of amounts of the product is also a declining exponential. What is the distribution of the summed lifetimes of these product molecules? We break up this distribution according to the number n of molecules produced and then sum over n to reach the actual distribution, with the contribution of each n weighted according to the likelihood of its occurrence.

The distribution of lifetimes for those cases in which a single molecule is produced ($n = 1$) is also exponential; for two-molecule cases ($n = 2$) it is not. To see this, note that the probability that two molecules will simultaneously decay at zero time is zero; the distribution of summed lifetimes will thus have a peak at nonzero times. As n increases, the distribution of summed lifetimes becomes increasingly sharp around n times the average lifetime (a classical statistical result), where we define the sharpness Q as the mean divided by the standard deviation. In making up the overall summed distribution, the weighting of each component decreases with increasing n so that the final distribution is again exponential (Fig. 1). In a linear system, this means that the distribution of the amounts of the enzymatic products of these molecules will again be exponential—and so on, up to and including the final open channels, so that the distribution of bump areas is also necessarily exponential.

We have been able to show that this argument is completely general for molecular linear chain systems each of whose active stages has only a single active state. An "active stage" is an enzyme, and an "active state" is an active energy state of this enzyme. If more than one state is active, the summed active life of the stage having the active states and, therefore, the distribution of numbers of products will no longer be exponential and, in fact, will have a peak at a nonzero value (by the argument given above). The peak becomes sharper with increasing number m of states. The sharpness Q of the peak is maximal for states of equal lifetime and activity and is then equal to $\sqrt{m}$.

We now outline the analysis, which will be given in detail elsewhere, that makes these considerations rigorous. The analysis starts with a description of the most general possible linear chain system based on molecular processes—including branching, linear loops, backward transitions, multiple active states, and deterministic amplifications (those in which the amount of product is fixed, not statistically variable). First, we studied the cases of unbranched systems

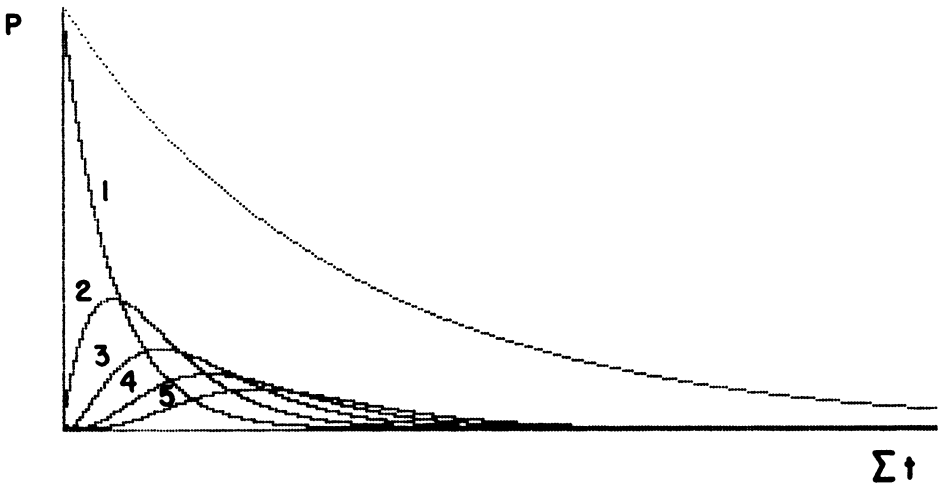

FIG. 1. The distribution P of summed lifetimes Σt of products of an enzymatic reaction in a linear chain model is composed of the summed lifetimes arising from each definite number n of product molecules. The sum over n is weighted by the probability of occurrence of each n. The lower curves in the figure are the summed lifetimes for 1–5 enzymatic molecules, with their areas weighted according to a discrete exponential distribution $q \cdot p^{n-1}$, with $q + p = 1$, where we have chosen $q = 0.17$ for this example. The upper curve is the sum over all n to infinity and is an exponential whose time constant τ is the time constant of the component distributions multiplied by 0.17.

(with no deterministic amplifications) each of whose enzymatic stages has only a single state. In this case, the activation of each stage by the preceding stage may be described as a Poisson point process with an average rate λ. Thus, if the distribution of the summed lifetimes of the parent stage is exponential with time constant τ, the distribution of the quantities n of the daughter will be $P(n) = \int_0^\infty (1/\tau)\exp(-t/\tau)\cdot(\lambda t)^n \exp(-\lambda t)(1/n!)dt$, which has the form $P(n) = q \cdot p^n$, where $q + p = 1$. This form may be called a "discrete exponential." For a given number of daughters, the distribution of the summed lifetimes is a gamma distribution. The exponentially weighted sum of gamma distributions is $P(t) = \Sigma_{n=1}^{\infty} q \cdot p^{n-1} \cdot t^{n-1} \cdot \exp(-t/\tau)/(\tau^n \cdot (n-1)!)$, which is an exponential. Thus, by induction, all stages (including the final channels and, therefore, the conductances) have exponential distributions for their summed lifetimes and, therefore, for their areas.

These conclusions are not modified if linear loops are included in the chain, provided each stage has only one active state.

The results are changed, however, if the chain includes branching, deterministic amplifications, or enzymatic stages with multiple active states. Since the treatments of all three cases are similar, we shall refer here only to the last. Here we define the area A of an enzymatic state as the product obtained by multiplying the mean rate λ at which the state produces its daughters by the time it lives t: i.e., $A = \lambda t$. But t is exponentially distributed, with time constant τ, and so is A, with area constant $a = \lambda\tau$. If a stage has m states all contributing to the area, with area constant a_i where $1 \leq i \leq m$, and $f_i(A) = \exp(-A/a_i)/a_i$ is the area distribution of state i, then the area distribution of the stage, $f(A)$, is simply the convolution of all partial state area distributions, that is $f(A) = f_1(A) * \ldots\ldots * f_m(A)$. We show by induction on m or by optimization methods that the maximal sharpness of f occurs when $a_1 = a_2 = \ldots = a_m$. The convolution of m exponential distributions with equal area constants a is a gamma distribution $(A^{m-1}e^{-A/a})/a^m m!$ whose sharpness can be shown to be independent of a and equal to $\sqrt{m}$. Thus, we show in the proof to be published that, if $m > 1$, the stage area distribution may have a peak at a nonzero value, that this peak is narrowest when the component states have equal average areas, and that the sharpness Q is then $\sqrt{m}$. We then show by inductively extending this last conclusion to a number of stages that Q of the final-channel area distribution cannot be greater than that of the sharpest stage. Therefore, an observed value of Q for the conductance area distribution requires that at least one enzymatic stage of the process have at least Q^2 active states.

If the amplification factor at some stage is sufficiently small that an appreciable number of failures occur (zero product molecules), then this conclusion applies to the Q calculated excluding the failures. If the failures are included, a lower experimental value of Q results but a stronger theoretical statement can be made: That the number of active states m of the *first* enzymatic stage must be greater than Q^2.

It is important to note that, unlike the area distribution, bump amplitude distributions can be nonexponential and even peaked, even for single-active state, unbranched linear chain models (3).

We measured the area distributions of bumps in *Limulus* ventral photoreceptors by standard voltage-clamp techniques (4). The data were recorded and the analysis was carried out on an Apple II computer. The inset of Fig. 2 shows a sample recording obtained during continuous weak illumination. The area of each bump was measured by the computer. Most bumps appeared to have similar time courses; bumps with strongly deviant time courses were rejected by the computer as double or multiple events. The number of rejected events fitted that predicted assuming an exponential distribution of interbump intervals. About 10% of the recorded bumps were rejected at this light intensity, and we estimate that 2% of the remaining measured bumps were multiple events. Histograms were obtained for bumps recorded during illumination and for bumps recorded in the dark, and a difference histogram is shown in Fig. 2. Similar shapes were obtained at half and twice the light intensity, with no detectable scale change, suggesting that no appreciable light adaptation occurs at these very low intensities. Only those histograms were used which were obtained while the average bump amplitude and the histogram shape did not vary with time.

The histogram of Fig. 2, and all others obtained from four other cells, do not depart significantly from exponential forms (χ^2 test). [A recent abstract by Goldring and Lisman (5) reports small but apparently significant deviations from exponentiality.] In some of the same cells, the amplitude distributions were strongly nonexponential and sometimes even had peaks at nonzero values.

Thus, *Limulus* passes the above strong test for nonmultiple-active-state linear systems. Although this does not of course prove linearity of the single-photon process, it sug-

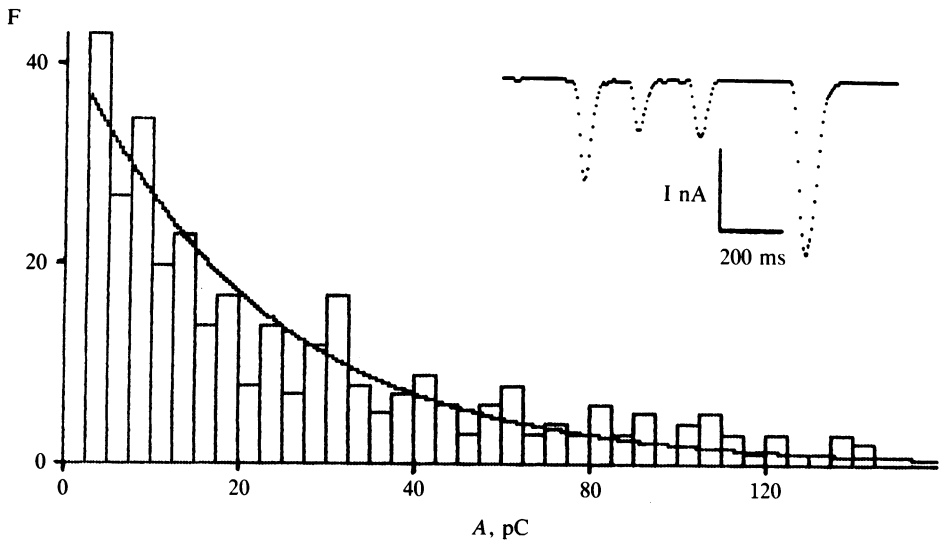

FIG. 2. Histogram of areas A (time-integrals of conductance changes) of bumps (single-photon responses) in a *Limulus* ventral photoreceptor, F being the frequency of occurrence. The histogram is the difference between that recorded in weak light (1.5 sec^{-1}) and in the dark (0.1 sec^{-1}). A total of 337 light bumps were recorded in this run. The curve is an exponential with the same mean (22.5 picocoulombs) and the same area as the histogram, and it is not statistically different ($\alpha > 0.2$, χ^2 test) from the histogram. Baseline noise and undetected bumps could have affected only the first column of the histogram, which has accordingly been omitted. (*Inset*) A sample voltage-clamp recording.

gests that it may be well described by a simple chain of first-order biochemical reactions together with ordinary diffusional processes, which are intrinsically linear. These processes can modify the kinetics but not the area distributions of the bumps.

The transduction process in *Limulus* ventral photoreceptors cannot, in fact, be totally linear. The responses of these photoreceptors to multiphoton stimuli are not simple sums of single-photon responses (1). This implies that there are interactions among the underlying processes, and therefore nonlinearities. These nonlinearities should manifest themselves in the single-photon processes but do not. Three types of nonlinearity appear in the multiphoton stimulus–response relationship (6): At high intensities, (*i*) a saturation, and at intermediate intensities (*ii*) an enhancement of the transient response and (*iii*) a depression of the steady-state response (light adaptation). Saturation: It is not surprising that saturation does not appear in the single-photon response. Adaptation: The very existence of a transient suggests that the onset of light adaptation is fairly slow (7) and the process may, therefore, not develop until too late to affect the bump area substantially. Enhancement: This effect is fast (unpublished results) but also relatively weak, and one must assume that it is too weak to affect the individual bump area appreciably.

Confrontation of published observations of single-photon responses in toad (9) and in locust (10) with the test described above leads to conclusions very different from those reached for *Limulus*. In both cases, measurements were not done under voltage clamp, but the smallness of the responses suggests that the conductance area distributions may not be grossly different from the voltage or current area distributions. The estimated sharpnesses (Q) of these distributions are 2.2 and 5 for locust and toad, respectively. The minimum numbers of active states in some stage are, therefore, 5 and 25, as compared with 1 in *Limulus*. These minima are approached only if the active states have comparable product amounts.

These numbers do not take into account failures (non-unity probability that an isomerization results in a bump). However, Fein and Szuts (11) summarize data suggesting that this probability is, in fact, ≈ 1. If it is assumed to be 1, the preceding conclusion applies not just to some stage but to the *first* enzymatic stage of the transduction process. Within present uncertain biochemical models, this implies that at least 5 or 25 states of rhodopsin would have to participate in the activation of the G-binding protein (12). It is unlikely that the transduction process has 25 active states in any stage; the process in toad must therefore contain strong nonlinear stages. In locust, the presence of 5 active states in a single stage is possible, but improbable.

What kinds of nonlinearities can convert a declining probability distribution of summed lifetimes of an enzymatic ensemble into a distribution peaked at nonzero product quantities? If the dependence of the amount of product on the total summed active lifetime of the enzyme is linear, the product distribution will be the same as that of the enzyme, as discussed above. If this dependence is supralinear, each range of summed lifetimes translates into a range of product amounts that increases with increasing summed lifetime. Therefore, the product amount probability curve is increasingly depressed for increasing amount; that is, the curve falls even more rapidly than the summed-lifetime curve and cannot be peaked. The opposite, however, is true for a sublinear dependence of product on lifetime, and a peaked product curve can result.

Processes that can result in linear or supralinear product/enzyme dependences only and which, therefore, cannot be responsible for the toad and locust observations include positive feedback, positive feed-forward, and positive cooperativity. Processes that can result in linear or sublinear processes only include negative feedback, negative feed-forward, negative cooperativity, and saturation (barrier and/or diffusion-limited substrate availability). Note that these processes in toad and locust must be significant at the level of single-photon responses.

A possible mechanism of nonlinear negative feedback is the inactivation of rhodopsin by phosphorylation by a light-activated kinase (12). Barrier-based saturation—that is, the existence of a limited anatomical substrate reservoir—has been suggested to arise from exploitation of all the phosphodiesterase or Ca^{2+} in a single rod disc (8, 13). In the invertebrate, the microvillus could provide a similar anatomical limitation.

We are grateful to Dr. Shaul Hochstein and especially to Dr. Bruce Knight for extensive discussions, to Dr. Alan Fein for hospitality in his laboratory (N.M.G.), and to Engineer Aryeh Adan for the voltage-clamp amplifier design. This research was supported by

a grant from the Fund for Basic Research of the Israel Academy of Sciences and Humanities.

1. Dodge, F. A., Knight, B. W. & Toyoda, J. (1968) *Science* **160,** 88–90.
2. Borsellino, A. & Fuortes, M. G. F. (1968) *J. Physiol. (London)* **196,** 507–539.
3. Tiedge, J. (1981) Dissertation (Aachen Univ., Aachen, F.R.G.).
4. Katz, G. M. & Schwartz, T. L. (1974) *J. Membr. Biol.* **17,** 275–291.
5. Goldring, M. & Lisman, J. (1983) *Invest. Ophtalmol. Vis. Sci.* **24,** Suppl. 178, abstr. 5.
6. Brown, J. E. & Coles, J. A. (1979) *J. Physiol.* **296,** 373–392.
7. Lisman, J. E. & Brown, J. E. (1975) *J. Gen. Physiol.* **66,** 473–488.
8. Yoshikami, S. & Hagins, W. A. (1973) in *Biochemistry and Physiology of Visual Pigments*, ed. Langer, H. (Springer, Berlin), pp. 245–261.
9. Baylor, D. A., Lamb, T. D. & Yau, K. W. (1979) *J. Physiol. (London)* **242,** 613–634.
10. Howard, J. (1983) *Biophys. Struct. Mech.* **9,** 341–348.
11. Fein, A. & Szuts, E. (1982) *Photoreceptors: Their Role in Vision*, IUPAB Biophysics Series (Cambridge Univ. Press, Cambridge, U.K.).
12. Liebman, P. A. & Pugh, E. N., Jr. (1979) *Vision Res.* **19,** 375–380.
13. Kuhn, H., Cook, J. H. & Dreyer, W. J. (1973) *Biochemistry* **12,** 2495–2502.