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Our Present Knowledge of Calcium or Hydrogen Ions as Transmitters in the Vertebrate Rod Outer Segments*

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Abstract. Difficulties in testing the possible role of calcium as a Transmitter in the R.O.S. are discussed. A comparison is made with the Sarcoplasmic Reticulum system where calcium flux are easily measured. The latest results reviewed on intact cellular structures are highly indicative but not yet conclusive.

Key words: Rhodopsin — Rods — Phototransduction — Calcium.

In the vertebrate rod outer segments, the disc membrane has no continuity with the outer cell membrane from which it originates. This implies that a signal has to be transmitted through the cytoplasm, from the disc membrane where it is initiated by the bleaching of one rhodopsin molecule, to the outer cell membrane where a decrease of the sodium conductance is observed. Hagins proposed in 1970 that the transmitters might be calcium ions released from the disc inner volume, and estimated that a few hundred ions released upon the bleaching of one rhodopsin molecule could be sufficient to generate a detectable change in the cell membrane polarisation.

A large amount of work has been invested by many groups during the last five years to try and test this hypothesis, but no clearcut proof of its validity seems to have been obtained as yet.

One might wonder why a simple proposition like this calcium hypothesis appears so difficult to check and then, if such is the case, why it is so important to work on this very system, the vertebrate rods, instead of concentrating on a photoreceptor system more accessible to experiments, such as found in invertebrate (limulus or barnacle). The major problem with the rods is the great difficulty of intracellular works and particularly of intracellular injections. These are, comparatively, much easier in some invertebrate photoreceptors. But beside the fact that vertebrate and invertebrate photoreceptor have opposite response to illumination (depolarisation in invertebrate and hyperpolarisation in vertebrates), the important point is that the

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vertebrate R.O.S. may be the only photoreceptor cell for which, when the transmitter is properly identified, the next step, that is the study of the molecular mechanisms leading to this transmitter release, can be undertaken. The vertebrate retina provides indeed unique advantages for the isolation of large numbers of photoreceptor cells, and the purification of large quantities of photoreceptor protein is easy. An alternative choice could be discussed, that of the squid or octopus retina, but the electrophysiology does not seem easier there than in the vertebrate rods, and the biochemistry is certainly less practical.

Now, why is an intracellular calcium release so difficult to characterise in the R.O.S.? Calcium fluxes are routinely studied in another intracellular system such as the striated muscle sarcoplasmic reticulum (S.R.). There are however basic differences which make inoperative for the R.O.S. techniques which are successful for the S.R.:

– the R.O.S. are highly sensitive and saturate at a very low level of illumination, when only about 1 in 10^5 pigment molecule is photoexcited each second. The large number of pigment molecules in a cell is necessitated only to increase the sensitivity, the rhodopsin molecules within a disc are not supposed to work in parallel.

– the total amount of transmitter stored to be released may be fairly small, due to the low level of saturation.

– a release is always more difficult to study than an active pumping, and it is the active pumping which is usually followed in S.R. studies, not the release.

In facts the active pumping of calcium by the rod discs, which is the first requirement for calcium to be a transmitter is about the only step that seems now proven, although the nature of the pump is still debated. According to the classical scheme of transmitter characterisation, the other requirements for calcium to be accepted as a transmitter are:

b) if artificially added into the cell, Ca should simulate the effect of light.

c) if suppressed or buffered in the cell, the cell should be desensitised to light.

d) light, at physiological level, should induce a release.

e) the time course of the release should be compatible with the physiological response.

Although a lot of data have been presented on point d), most of them have been obtained on broken cells or even isolated membrane vesicle preparations, and use strong illumination, bleaching a large percentage of the rhodopsin molecules. Changes of calcium passive efflux have been observed this ways, but this could be due to non specific changes of the disc membrane properties, induced by the modification of such a large proportion of the proteins within the membrane, and by the release of a large number of free retinal molecules. Observations of calcium efflux after a weak illumination have not been definitely confirmed with such preparations, after sometime over optimistic preliminary reports.

Results however have been obtained on points (b) and (c) on intact physiological preparations by two approaches. Hagins and Yoshikami use external electrodes to measure external current gradients along the rod. Knowing that extracellular calcium was already simulating the effect of light, they first used calcium ionophore to equilibrate intracellular and extracellular calcium levels [1]. A suppression of the dark current was then obtained with much lower extracellular calcium concentra-

tion. In a more recent experiment [2] they introduced in the R.O.S. calcium buffers. The method used is the fusion with the cell outer membrane of phospholipid vesicles (liposomes) preloaded with calcium buffer. The simultaneous loading of the liposomes with a fluorescent dye allowed the control of the amount of calcium buffer really entering the cell. Proton buffers have been found ineffective but calcium buffers do cause a reduction of the light sensitivity of the cell: a larger number of photon is necessitated to totally suppress the dark current.

Direct intracellular injection of calcium by microelectrode technique has been recently obtained by Pinto et al. [3]. The injection is slow, the amount of calcium injected cannot be controlled exactly, and the cell response is measurable a few seconds only after the injection. However, a definite hyperpolarisation is observed after the intracellular injection.

Points b) and c) of our list of requirements may therefore be considered as well supported experimentally. No good data are yet available on points d) and e). This will probably come when one succeeds in injecting intracellularly the calcium sensitive dye Arsenazo III, and in repeating in the R.O.S. the type of meaurement performed by Brown et al. on the limulus photoreceptors [4].

Now one should keep in mind that a calcium transmitter does not necessarily means that rhodopsin is a light sensitive calcium ionophore. Owing to the great lateral mobility of the rhodopsin molecules within the disc membrane one can imagine that there are fewer calcium channel molecules, one per 10^2 or 10^3 rhodopsin. The channel molecules would be opened by a random collision with a bleached rhodopsin molecule, the number of such collisions being in the range of 10^5-10^6 per second [5]. Such a process is already implied in the activation of cGMP phosphodiesterase by the bleached rhodopsin: Miki et al. [6] have shown that one molecule of bleached rhodopsin can activate one molecule of phosphodiesterase, although there is only one molecule of phosphodiesterase for 900 molecules of rhodopsin in the disc membrane. In fact the involvement of cGMP as an intermediate in photodetection has been discounted on the basis of wrong time estimates: at low levels of illumination visual excitation occurs within hundreds of milliseconds and not milliseconds as quoted by Miki et al. However this factor of 10^2 in the time scale seems still a little short to take cGMP as a good candidate. It remains that cGMP is up to now the only small diffusible molecule, whose concentration within the rod cell has been proven to be sensitive to very low illumination. Precise kinematic data would be very interesting to get on this process.

The most evident change observable when bleaching rhodopsin in the membrane, letting alone the spectroscopic changes, is the proton uptake in the stage Meta I $\rightarrow$ Meta II. The fundamental significance of the similar step in the bacteriorhodopsin bleaching cycle, and the general importance of transmembrane pH changes in bioenergetic processes may suggest that it is also here an important step in the generation of the signal by rhodopsin. It is however unprobable that the transfer of one single proton could directly, without another amplifying mechanism, transmit a signal from the disc to the outer cell membrane.

Two types of arguments may be raised. First, one would like similar processes to occur in cones and rods. A mechanism whereby the transfer of one single charge through the disc membrane triggers the release of transmitter would not work in cones, due to the continuity of the disc membrane with the plasma membrane. But

even in the rods, consideration of signal to noise ratio tend to reject this simple hypothesis.

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Discussion
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I would like to call back that in the two systems we have investigated, namely *first* dim, constant room light and incubated cattle retinae *second* weak flash illumination of superfused frog retinae, significant changes of the cyclic GMP content were seen at the earliest time, at 5 and 3 s, respectively.

In addition we have shown that maximum responses are not yet attained after the shortest time intervals.

For that reason and as long as more sophisticated techniques are not available for the measurements of the retinal cyclic GMP content in the subsecond range, we tend to favour the possibility that cyclic GMP acts as a modulator of the photoreceptor excitability rather than as an "internal transmitter".