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A historical perspective on the lateral diffusion model of GTPase activation and related coupling of membrane signaling proteins

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Aspects of the discovery of lateral diffusion of the G protein coupled receptor (GPCR) rhodopsin and that a single activated rhodopsin can non-covalently catalyze GTP binding to thousands of GTPases per second on rod disk membranes via this diffusion are summarized herein. Rapid GTPase coupling to membrane-bound phosphodiesterase (PDE) further amplified the signal via cGMP hydrolysis, essential to visual transduction. Important generalizations from this work are that biomembranes can uniquely concentrate, orient for reaction and provide a solvent appropriate to rapid, powerful and appropriately controlled sequential interaction of signaling proteins. Of equal importance to function is the timely control and termination (quenching) of such powerful signaling amplification. Downstream modulation by GTPase activating proteins (GAPs) or Regulators of G protein signaling (RGS) and related mechanisms as well as limitations set by membrane domain fencing, structural protein binding etc. can be essential in relevant systems.

The very interesting question that you raise of whether a single GEF might catalytically activate multiple, diffusible copies of Ras superfamily G'ases on cellular membrane surfaces is of course presaged by my discovery in the late 70s early 80s of precisely such a mechanism that is required for the activation of the heterotrimeric G protein, transducin by the GPCR, rhodopsin, in visual transduction. At that time, we found that a single activated rhodopsin molecule, could activate thousands of copies of G'ase through lateral diffusion-mediated collisions between those proteins on rod disk membranes (1–4). In turn, the activated G'ase molecules encounter and activate cGMP phosphodiesterase molecules bound to the same membrane. Our discovery that a single activated GPCR is an enzyme that catalyzes rapid GDP-dissociation/GTP-binding to multiple copies of G'ase encountered in its ongoing Brownian travels at the rod disk membrane surface was completely novel and unexpected.

As our unique experimental evidence gradually convinced me of the certainty of this surprising GPCR signal amplification mechanism some 35 y ago, I immediately wondered as you do now, about the generality of such a lateral diffusion-mediated, serial activation mechanism for other signal transducing systems.[1,2] But if lateral diffusion of membrane and peripheral proteins on membranes is a general property of cell membranes as we now know it to be, receptor-mediated amplified G'ase activation in other systems simply MUST be true. Of course, it is possible that lipid rafts, membrane protein fencing, cytoskeletal structures etc. might be designed specifically to prevent excess amplification. But wouldn't that be equally interesting to know, to understand and to control? So, in my view, it is still legitimate, even essential, to ask for every such membranous signaling system, whether lateral diffusion is a part of the activation and control mechanism; and especially including the possibility that single receptors (GEFs) may similarly use membrane surfaces to mediate multi-copy activation of the many RAS family G'ases and their effectors and/or GAPs.

What allowed these breakthrough discoveries in visual transduction was our ability to precisely limit the number of rhodopsin molecules activated while at the same time kinetically recording and quantitatively counting molecules of activated transducin and phosphodiesterase during R* and GTP'ase life-cycles of single experiments. This accomplishment was, of course, immeasurably aided by the two stage GTP-binding/PDE enzymatic design of the native biochemical amplification process itself (see below). Some of the relevant numbers for cognate parts of your GEF/G'ase systems may already be known but experience warns me of the difficulty of making measurements similar to ours under necessarily more complex in situ conditions so I am not surprised if we do not yet have clear answers to these questions today.

Though quite different experimental tools may be required to evaluate these mechanisms for GEF/Ras families, it may be worthwhile to examine some of the implicating features that made this fascinating physical/biochemical/diffusional mechanism discoverable in visual cells.

First, vision is a function of the eye, an organ readily studied at multiple levels in animals and man. Second, eyes are easily obtained in purity for physiological/biochemical study from animals and in mass, from abattoirs. Third, vision cannot occur

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without absorption of light. The only colored material able to perform this function, the carotenoid GPCR ligand of the visual pigment, rhodopsin etc. is contained within an outer retinal monolayer of cells, the rods and cones where visual transduction occurs. Fourth, there are about 100 million rods arranged at this uniquely accessible outer surface in most vertebrate retinas. The outermost portion of these, the outer segments that contain all the transduction machinery practically prepurified in situ, are readily detached from their fragile cilium connection to the cell body and collected.

Ready availability of material however did little to speed our search for mechanism until more global understanding gradually clarified what properties we should be looking for to explain vision at the biochemical/biophysical level. These properties turned out to be signal sensitivity, response speed and amplification (gain, speed and sensitivity) and only later, mechanisms that might modulate those properties.

Eye and Molecule Sensitivity: Beginnings of the picture were contributed by the fundamental physical analysis and experiments of Hecht, Shlaer and Pirenne[5] who proved that single photons activate human vision. Subsequent workers added proof beyond the shadow of doubt. A single photon can only be absorbed by and activate a single molecule[6] and that molecule was early identified as rhodopsin by the match of its visible light absorption spectrum with the action spectrum of vision. Moreover, the high (0.65) quantum efficiency of vision[7] was subsequently determined to match that for the bleaching of rhodopsin.[8] Rhodopsin had already been purified from retinas[9] and was early intuited to be an integral membrane protein because of the requirement of chaotropic agents (bile salts, later detergents) for its solubilization/extraction. Thus, one could say that the idea that visual transduction starts in a membrane protein might have been justified long before membrane proteins or signal transduction was even defined!

Electrical Sensitivity: The electrical consequence of this remarkable single photon particle sensitivity is the cell membrane "quantum bump" first looked for and finally recorded by Yeandle[10] in Limulus eyes and later found in vertebrate rods.[11] Nobel Laureate, Keffer Hartline, Yeandle's preceptor at Johns Hopkins who was the first to adopt Limulus for laboratory study, was also the first to adopt the brand-new technology of infrared night vision scopes. This made it possible shortly thereafter for early investigators including me, to actually see our experiments for the first time in complete darkness, without their "seeing" us. With light as the experimental stimulus, it is not a good idea to be working in a lighted laboratory! Our work would have been virtually impossible without such night vision equipment.

Amplification and Speed (gain and bandwidth): I worked for a number of years with Bill Hagins, an extraordinary NIH MD/physicist/mathematician/electrophysiologist, who brought novel biophysical approaches to bear on the problem of visual transduction. Hagins knew, respected and used his predecessors' shoulders well. Hagins' work formed an important stepping stone in mapping the reductionist problem for us through his fundamental physical thinking and imaginative experiments. From his work, we who listened finally understood what properties we must confirm to explain this miraculously rapid, single photon sensitive process capable of activating whole cells and causing synaptic transmitter release at the end.

Thus, using electric field gradient analysis before the invention of single cell suction electrode and ultrafine intracellular recording, Hagins' group[12] found rods to be hyperpolarized rather than depolarized by light (similar results were obtained by Tomita in Japan), measured the membrane current and charge flow per photon and showed that the response was local to the site of photon absorption. Whatever the coupling mechanism was to be, it would have to be ultimately sensitive (to single photons), very fast (vision sees the flickering of light from 5 to 80 Hz), extraordinarily amplified (to effect membranes quite distant from the photon absorbing molecule while diluted by oceans of cytoplasm in the way!) and function by blocking Na^+ channels rather than by opening them.

For many years, the odds on favorite mechanism for such requirements was Hagins' calcium hypothesis[13] i.e., that rhodopsin might be a rod disk Ca channel whose light activation allows rapid outflow of calcium ions into the cytoplasm from sequestration sites inside the disk, just as does sarcoplasmic reticulum activation in muscle contraction. This mechanism could have the sensitivity, speed and amplification needed. Although Ca^{2+} did turn out to have a role, it was not the central one that we discovered shortly thereafter in the cyclic nucleotide control mechanism that did have the requisite properties and whose modulation is now studied in such elaborate detail. At last, we found the properties that we had been looking for.

So what really makes these membranes and lateral diffusion so fundamental to biological function? In a nutshell, it is that membranes can concentrate relevant protein enzymes within and at their surface in optimum geometric orientation for reaction while at the same time providing the fluid vehicle for their rapid concentration-dependent interaction. In brief, they can assure and accelerate biomolecular reactions through confined diffusional encounters on the small distance scale relevant to regional specializations of cells. We could of course only gradually learn this one step at a time.

My own role in the discovery of lateral diffusion of rhodopsin and its enzymatic role in rapid amplification of G protein activation, began with my post-doctoral project with Britton Chance, where I designed and build ultrasensitive microspectrophotometers (MSP) that could quantify visual pigments in situ in single visual receptors.[14] Our original purpose was to determine what color vision pigments might be present in retinal cones. I became expert in the measurement and control of light and in understanding photon and electronic noise. During subsequent summers at Woods Hole's MBL, Bill Hagins educated me in the related electronic communication character expected of visual receptors (and indeed of all receptor-effector-modulator chains). I was also the lucky beneficiary of a convergence of the right background, perseverance and timing to evolving work of Sutherland, Rodbell, Gilman, Greengard and others, who were making seminal discoveries about hormone receptors, G proteins, cyclic nucleotides, protein kinases and their effects on cellular physiology. An almost incredible convergence of

those antecedent discoveries with our searches made it possible to rapidly determine the variants of these mechanisms that explained signal transduction in visual receptors.

The following is my historical perspective of the evolution of models of membrane structure, membrane fluidity, receptor activation, and G protein biology that played out in our current understanding of cell signaling by G proteins and allowed us to penetrate key aspects of the visual signal transduction cascade that quickly became an early prototype for G protein signaling. It really all began with my creation of those novel microspectrophotometers (MSP) needed to measure the visual pigment content of single cells without the need for detergent extraction and without concurrent destruction of the pigments by the light used to measure them.

1. Rhodopsin was known since the 1930s to be an integral membrane protein, only removable and purified in micelles from retinas using detergent solubilization.[15] In fact, rhodopsin was the first known receptor protein! Its initial purple color was early discovered to be "bleached" by light into the colorless metarhodopsin II, a product subsequently found to be the actual active end product of light action on the rod visual receptor pigment.[16]

2. Visual cell membranes: Vertebrate photoreceptor cells are long cylinders whose outer segments contain a cylindrical stack of 500 to 2000 of orthogonal Golgi-like flattened membrane saccules (lamellar disks). These disks, in rods, are separated from the ion channel-containing plasma membrane and are optimally designed to absorb longitudinally propagating light via the massive number (10^7-10^9) of visual pigment molecules embedded in them. An outer segment attaches to the rest of the cell via a single cilium from which it is easily broken away. We collected these in physiologic media in complete darkness and mounted them for study by MSP as they lay on their long cylindrical axes in the plane of a microscope slide.

3. Visual pigment is bleached by absorbed light (lose its visible spectrum) with near 100% efficiency. Both rod and cone cells are microscopically small in the service of spatial resolution of vision and therefore contain only a tiny amount of visual pigment. To measure the presence of such tiny amounts of photosensitive visual pigment (GPCR) molecules by MSP,[14] we had to use extremely dim monochromatic light and the extraordinarily sensitive MSP instrument that I developed. Our MSP spectra showed that rhodopsins are embedded in a highly oriented manner in the outer segment membranes[17]; preferentially absorbing only light whose electric polarization is perpendicular to the cellular cylinder axis (within the disk membrane planes). The ability of rhodpsin to maintain such preferential orientation in disk membranes is consistent with its integral structural role as part of those membranes as well as explaining the efficiency of physiological light capture for vision via optimum orientation of its chromophoric electric vectors.

4. Proteins in lipid: My MSP measurements on single rods found ca. 3mM (!) rhodopsin in a 2-D (liquid) crystalline lattice, a molecular density of ca. 30,000/μm^2 on the disk membrane surface.[17] Such packing density places adjacent rhodopsins only about one rhodopsin diameter apart and leaves room for only about one boundary layer of lipid molecules per rhodopsin (2 lipid diameters between adjacent rhodopsins).[1] Known rod peripheral proteins like G protein, cGMP phosphodiesterase etc. must be attached to these membranes by lipid embedment between the rhodopsins.

5. Discovery of a protein motion: MSP of most cone cells required use of ~1μm wide optical microbeams. Amphibian rods can be substantially larger; over 10μm in diameter. We repeatedly noted more rapid bleaching by the 1μm measuring light in smaller cells and less rapid pigment loss when using the same sized microbeam in larger cells.[18] This was puzzling for some time (there was no hint at that time that proteins could move around in membranes) until we gradually realized that molecules being bleached by small microbeams in large cells might in fact be getting replaced by molecules moving from the still-dark portion of each cellular disk membrane. This "preserving" effect vanished when cells were prepared in glutaraldehyde-containing media as expected if previously mobile molecules were being cross-linked.

6. Lateral diffusion: To determine the speed of this diffusional pigment movement, we needed to rebuild our microscope to allow microbeam flash photolysis on one side of the cell while measuring at other locations within the cell. These studies showed that rod visual pigment moves within seconds in the planes of the lamellar disc membranes[18] We used these data to calculate a lateral diffusion coefficient of about 0.5μm^2/sec for visual receptor pigment motion. We also determined that the previously puzzling beam-width dependence of bleaching speed was only laterally directed (side to side in the rod cylinder) and did not occur in the longitudinal (axial) direction. We called this "lateral diffusion." At this time, no one had any idea that rods were also replete with G proteins and other signaling proteins.

7. Gain/amplification: Several years later, in separate experiments designed to test for Ca^{2+} release that might mediate light activation of rods, I discovered instead the near-instantaneous light activation of cyclic GMP (cGMP) hydrolysis[19] that led rapidly to our understanding that rhodopsin is an enzyme that causes many copies of its substrate, transducin or G_t, to become activated and in turn, to activate many copies of cGMP phosphodiesterase (PDE).[2,3] Initially, though activity required GTP cofactor, we were measuring only PDE activity via loss of cGMP. But our further development of fast filtration and reaction quenching methods allowed us to simultaneously titrate the speed and magnitude (amplification gain) of radiolabeled GTP binding vs light intensity directly.[4]

8. Speed: Our experiments were by design, all kinetically resolved. We early noted[2,3] that onset speed of PDE activation became faster and faster with brighter flashes (more bleached rhodopsin) and slowed down to a limiting onset speed as the light was made dimmer and dimmer. These activity onset curves were always precisely parabolic ($A = kt^2$) with characteristic delay time of a couple of seconds to reach maximal PDE activity after very weak bleaches. We quickly noted that this timing corresponded closely to my earlier measurements of rhodopsin lateral diffusion speed as well as to the theretofore puzzling parabolic time delay of rod electrical responses to single photons.[11]

9. Membrane real estate: We performed quantitative analysis of complete light titration curves of PDE velocity and of GTP

binding.[4] The "half maximum" activity saturation of these curves showed that single photons were activating membrane regions (domains) near the size of an entire rod disk membrane. But final activity was always parabolically delayed[2,3] by a constant time for all the weakest lights below a limiting level comparable to the time it would take a single activated rhodopsin to carry activation to other proteins via its lateral diffusion.

10. Signal transduction via lateral diffusion mediated amplifier of vision: The size of single photon activation domains that we determined together with their congruent parabolic activity delay corresponding with both lateral diffusion and receptor electrical response delay times at the weakest light stimulus, thus gave birth to our R*G* lateral diffusion amplifier hypothesis of vision.[1-4] We had found that PDE activation and its preceding G protein activation had the single photon sensitivity, the gain/amplification of coupled rapid enzymatic activation, and the parabolic activation delay time (speed) of lateral diffusional reaction partner mixing consistent with the sought after parameters of visual transduction.[20]

11. The role of stoichiometry of components: We found the number of activated G proteins to exceed the number of activated PDE's on disk membranes by about their known static concentration ratio (~5:1). A 5-fold stoichiometric excess of GTPase over PDE would increase the yield of an intrinsically weak stoichiometric binding between G* and P on the membrane surface.

12. Reaction speed: The 5:1 ratio of G*/P would simultaneously decrease the time needed for activated GTPases to collide with PDEs by 5-fold if proteins diffused at comparable speed while fixed to the membrane surfaces. Thus, diffusional delay between R* and P activation would be only trivially longer than that for G itself. G and P are peripheral membrane proteins inserted largely via their long chain fatty acids. At the time of our discoveries, such peripheral proteins were speculated to be able to accelerate their lateral diffusion via "skating" or "hopping" off and onto the membranes. We proved that this is not the case in visual transduction.[21] Free diffusion rates for various lipids and lipid-attached peripheral proteins have been found to differ little from that of rhodopsin except in cases where structural proteins and fencing mechanisms can interfere, such as those found in red cell membranes (spectrin, etc), that are designed to retain membrane proteins in functional, diffusion domains smaller than the order of 1μm^2. In retinal rods, R* diffusion over 1um^2 can yield 3–6x10^3G*/R* as we found experimentally.

13. Membrane Essentials: Our lateral diffusion and signal transduction work provided an essential link at the dawn of recognition that biological membranes are 2-D liquid crystals[22,23] where membrane proteins can be concentrated and permanently held in a molecular orientation functionally important to chemical reaction with other components while retaining rapid planar motion within the liquid. Membranes orient proteins and other components optimally for more efficient bimolecular reactions both within the membrane and from the aqueous phase. During the first years when I was doing these measurements, people were still speaking of membrane proteins as if they were stationary peanut butter, spread sheet-like over the tops of the lipid molecules. Thus, our work on large amphibian rods where disk membranes are wide enough to permit placement of more than one microbeam of measuring and interrogating light in MSP, were the first to show in live continuous real-time that a receptor protein is in motion and to measure its speed as globular protein[18] embedded in lipid.

14. GPCRs are enzymes: Our discovery that R* is an enzyme capable of catalyzing GDP/GTP exchange with a turnover number of several thousand sec^{-1} to powerfully amplify a weak biological signal[2-4] was also the first recognition of a non-covalent mode of action by an enzyme. Until this work, enzymes were understood to mediate only covalent changes between substrate and product.

In conclusion, I agree with Vadim and Marie that there is need for subsequent time and amplitude sculpting mediated by other proteins to explain the refinements of visual transduction in circumstances that need more time resolution and less amplification in brighter light or in those that require enhanced statistical properties of signal and noise for reliable single photon detection in dim light. Such modulation is consonant with the need for appropriately rapid action to capture or escape other organisms for survival in bright light in addition to seeing sensitively and reliably in near-darkness.

Vadim mentions his later role in confirmation of my earlier discoveries of the extraordinary magnitude of R*-G'ase amplification. Such confirmations are always expected in science. None of this diminishes in any way the importance of the completely unexpected and extraordinarily rapid amplification of G protein activations resulting from single photon activation of a GPCR molecule, rhodopsin, that I discovered ~35 y ago and that still amazes me to this day. *I found that a single activated rhodopsin molecule is a non-covalent enzymatic amplifier, using the speed of short range lateral diffusion to encounter and activate thousands of copies of the G'ase, transducin, on a membrane surface.* The necessity for the properties of immense amplification, ultimate sensitivity and maximum speed in visual R*/G'ase/PDE coupling and its absolute dependence on membrane lateral diffusion for expression of these essential properties that we discovered, underlies everything! Moreover, these same physical properties are intrinsic to all biological membranes. Such properties clearly need to be reckoned with for every membrane protein system. They are the *sine qua non* of our GPCR membrane signal transducer mechanism.

In this selective historical perspective on some of my early work that contributed to our most fundamental models of biological membranes including lateral diffusion on/in a membrane, signal amplification, and non-covalent enzyme actions, I have tried to highlight those aspects that address the question posed. Simply, can a single GEF activate more than one G'ase and is there a pool of resultant freely diffusible, activated G protein that leaves the receptor to encounter its effector/G'ases? In the case of the photoreceptor system I can answer definitively, yes. As sagaciously addressed in the accompanying article from Eliot Ross, it is still uncertain to what extent the rhodopsin/G_t system is a perfect model for other receptor-G protein systems. But I argue that the principles discovered through our work and that of others on the photoreceptor system are certainly relevant to all.

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