Stochastic trigger for clathrin-coated vesicle biogenesis

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ABSTRACT

Kinetic aspects of receptor-mediated endocytosis are identified, particularly as relating to the stochastic formation and released of clathrin coated vesicles at the plasma membrane. We discuss how one might account for the coordinated steps of this process, including receptor activation, association with adaptor proteins, coat formation, and the role of phosphoinosotide metabolism as a regulatory mechanism. In anticipation of building a detailed mathematical theory, we discuss an earlier treatment of the way threshold fluctuations affect the firing probability of nerve axons (H. Lecar, and R. Nossal, *Biophys. J.* **11**, pp. 1048--1067, 1971), in which equations were analyzed by distinguishing different time-scales to identify pertinent kinetic variables

Keywords: endocytosis, clathrin coated vesicles, phosphoinositides, fluctuations

1. INTRODUCTION

Many substances--including receptors for growth factors and other signaling molecules, cholesterol-rich LDL complexes, iron and other critical metabolic factors, and certain viruses--enter eukaryotic cells from their surroundings by a process known as receptor-mediated endocytosis.[1] The latter involves the formation of a multi-component coat on the plasma membrane, a major element of which is a protein heteropolymer known as a "clathrin triskelion." The triskelions assemble into a geometrically distinctive lattice that plays a mechanical role in vesicle biogenesis but, more importantly, also binds a complex array of ancillary proteins that interact with lipids and other components integral to the membrane. These clathrin-associated proteins act in concert with yet other cellular constituents in events leading to cargo sequestration, coat formation, membrane invagination, vesicle scission and, ultimately, delivery of the vesicle and its contents to internal cell compartments where the cargo is liberated. The formation and release of a clathrin-coated vesicle ("CCV") from the plasma membrane can be considered as a discrete process that signifies a successful outcome.

However, oftentimes after the cell is stimulated (e.g., by supplying cargo), CCV formation is seen to begin, but coat formation and vesicle budding fails to go to completion. The nascent vesicle regresses and the system reverts to a "rest" state, where evidence of an assembled coat at that site vanishes. Thus, receptor mediated endocytosis is like other biological phenomena where, once certain thresholds in stimulus are surpassed, the state variables describing macroscopic observables take values that are distinctly different than their initial measures. In several instances these events have the appearance of being related to two- or multi-state switches having discretely different outcomes. Perhaps the best studied of these is the generation of an action potential that results in signal propagation along a nerve axon.[2] Other examples are bistability in bacterial populations,[3] the transition from latent to lytic states of host-incorporated bacterial viruses,[4] mitochondrial-dependent apoptosis ("programmed cell death"),[5] and switching of carbon metabolism in bacteria.[6,7] In several instances it has been demonstrated that the switching characteristics of these and similar processes are mediated by the presence of "noise" related to fluctuations in either internal cellular events or external signals.[8-10] We add clathrin-coated vesicle formation to this list, considering it as an excitation process having two outcomes characterized by a stochastic transition between success and failure. Like nerve excitation, CCV formation entails a complex kinetic interplay between several significant system variables.

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CCV formation first interested electron microscopists, who noted polyhedral features on the surfaces of vesicles budding from the plasma membrane of various cells. The material responsible for these structures subsequently was isolated and named clathrin because of its ability to form cage-like entities in vitro. Clathrin is a protein dimer composed of a *ca.* 590 kDa heavy chain and a variable 23-35 kDa. light chain[1,11] found in a cell as a three-legged pinwheel (see Fig. 1) which assembles into nets on the cytoplasmic side of the plasma membrane. Under certain circumstances these nets are flat and appear, essentially, as hexagonal lattices. Usually, though, the triskelia form bent polyhedral baskets whose facets, in accord with topological rules,[12] are hexagons and pentagons. Although clathrin can assemble into such baskets in vitro either with or without associated "assembly proteins" (depending on solution conditions), it forms regular structures on membranes only if various other proteins are present. More than 200 proteins have been identified as potential clathrin binding partners,[13] although only a fraction are expressed in any particular cell type. These clathrin-associated proteins can be categorized as falling within several classes that illustrate their function:[14] proteins that bind clathrin to receptor complexes that span the plasma membrane, the receptors often being bound to ligands that are to be internalized; proteins that bind to selective membrane lipids and impart curvature into the membrane; the previously-mentioned assembly proteins that facilitate clathrin lattice formation; proteins that bind to completed vesicles to effect their release from the membrane; proteins involved in directing the coated vesicles away from the plasma membrane towards endosomes; and, other proteins that bind with the coat to cause its dissolution so receptors and cargo can be liberated.

Although the exact sequence by which these events occur is not yet entirely clear and the overall process of CCV formation clearly is very complicated, we herein attempt to discuss these activities with a view of ultimately developing a comprehensive mathematical framework. We also consider how phosphoinositide metabolism may be involved, and indicate how the stochastic nature of CCV formation can be linked to various sources of noise that affect the response of a cell. First, though, we review a similar problem pertaining to the stochastic firing of space-clamped axons,[15, 16] the analysis of which resulted in expressions that relate the probability of nerve firing (production of an action potential) to both the characteristics of a stimulus and the sources of noise acting on the axon during excitation. This treatment serves as a guide for our subsequent discussion.

2. THRESHOLD FLUCTUATIONS IN NERVES

The treatment of threshold fluctuations in nerves[15,16] can serve as a paradigm for treating noise in other triggerable biological systems. A set of deterministic equations, the Hodgkin-Huxley equations,[2] had been established to account for axonal response to stimulation and their parameters had been well characterized. Thus, the addition of noise was a perturbation that affected their solutions but, in principle, the changes brought about by the noise terms could be accounted for by analytical theory.

The empirical equations derived by Hodgkin and Huxley for a space-clamped squid axon have the following form:

$$C\frac{dV}{dt} = I - I_i, \tag{1}$$

where I is an external stimulating current and I_i, the ionic current across the membrane, is given by

$$I_i = g_K n^4 (V - V_k) + g_L (V - V_L) + g_{Na} m^3 h (V - V_{Na}) \tag{2}$$

and the first term on the r.h.s represents the potassium current, the second represents the chloride leak current, and the third represents the sodium ion current. The variables n, m, h obey equations of the form

$$\frac{d\nu}{dt} = \tau_\nu^{-1}(V)[\nu_\infty(V) - \nu]\ ;\ \nu = n, m, h \tag{3}$$

where $\tau_\nu(V)$ and $\nu_\infty(V)$ are a voltage-dependent time constant and a voltage-dependent steady state value for the parameter ν, where $\nu_\infty \equiv 1$. The quantities given in Eqs. 3 are highly non-linear functions of the voltage,[2] so Eqs. 1--3 comprise a complicated, 4-dimensional system that, at first, looks to be impossible to manipulate analytically. However, the timescale for changes in m, which pertains to the opening of the sodium conductance has an associated timescale $\tau_m(V)$ that is approximately 10 times faster than that of the variables h (the closing of the sodium conductance) and n (the opening of the potassium conductance). Consequently, if one fixes n and h at their rest values and numerically solves the remaining 2-dimensional system (the Vm equations), one finds that the early time solution is close to that of the entire Hodgkin-Huxley (HH) set for equivalent times.[15] The difference is that there are 3 steady-state solutions of the Vm set (those values of V, m for which $\frac{dV}{dt}$ and $\frac{dm}{dt}$ are zero), whereas there is only one steady-state for the full HH set. Two of the Vm singular points are stable fixed points, one signifying a "rest" state ("A" in Fig. 2), and the other ("C") an "excited" state, which corresponds to the full HH action potential. That is, because in the system the variables n and h are chosen to be constant, the system lacks a mechanism for recovery and the excited state is a constant plateau value of $V(t)$.[15] (The full HH system indicates that the nerve returns to its rest state after the action potential excursion.) The third singular point (designated as "B" in Fig. 2) is an unstable saddle point, which we here refer to as the "threshold singular point," as it anchors a limiting trajectory, the "threshold separatrix," (dotted line in Fig. 3) that divides the trajectories in the Vm phase plane into two classes. In the absence of "noise," the phase points lying on trajectories located to the right of the separatrix always terminate in the excited state, whereas those on the left return to the rest state. (The latter correspond to excursions that do not result in an action potential when the axon is stimulated, being well captured by solutions of the full HH equations.)

In the case of in vitro axonal stimulation, noise is linked to material fluctuations arising because the preparation is at finite temperature. One source, for example, is Johnson Noise that is present in an electrically resistive element; another possibility is conductance noise linked to the thermal fluctuations of discrete transport channels.[17] How can such perturbations be handled within the context of the Vm equations? If we assume that the noise is additive, then Eqs. 1-3 for the Vm system can be written as

$$\frac{dV}{dt} = J - \gamma_0(V - V_0) - \gamma_1\sigma(V - V_1) + F_V(\langle\sigma\rangle, t) \tag{4}$$

and

$$\frac{d\sigma}{dt} = \phi(V, \sigma) + F_\sigma(\langle V\rangle, t), \tag{5}$$

where $\gamma_o, \gamma_1, J, V_0, V_1$ are constants related to $g_K, g_L, g_{Na}, C, h_\infty(V_{rest}), n_\infty(V_{rest})$, etc., as explicitly given in Ref. 11. The variable σ is the conductance which for the HH treatment of the squid axon is $\sigma \equiv m^3$, and $\phi(V, \sigma)$ is a function chosen to mimic the properties of the Na conductance. The Langevin forces F_V and F_σ are random time functions that, in general, can depend on the mean value of the voltage $\langle V(t)\rangle$ or conductance $\langle\sigma(t)\rangle$. Equations 4 and 5 are then expanded about the values V_B, σ_B at the threshold singular point, viz., in terms of $\varepsilon \equiv (V - V_B)/V_1$ and $\mu \equiv \sigma - \sigma_B$, yielding two coupled

linear equations in ε and μ, viz., $d\vec{x}/dt = \underline{\underline{A}} \bullet \vec{x} + \vec{F}_x(t)$, where $\vec{x}$ is a vector whose elements are ε and μ, $\underline{\underline{A}}$ is the appropriate matrix of coefficients, and $\vec{F}_x$ is a vector representing linearized noise terms (with suitable coefficients). If one then transforms the variable according to $\vec{y} = \underline{\underline{Z}} \bullet \vec{x}$ such that the matrix $\underline{\underline{A}}$ is diagonalized (the "principal axis transformation"), one readily obtains

$$y_i(t) = y_i(0)\exp(p_i t) + \int_0^\infty \exp(p_i(t-s))[Z_{i1}F_\varepsilon(s) + Z_{i2}F_\mu(s)]ds \tag{6}$$

where the rate constants p_i are the eigenvalues of $\underline{\underline{A}}$.

The problem now is reduced to a one-dimensional walk in the y_1 direction, yielding as solution

$$Y(t) \equiv y_1(t) - y_1(0)\exp(p_1 t) = \int_0^t \exp(p_1(t-s))X(s)ds, \tag{7}$$

where $X(t) = Z_{11}F_\varepsilon(t) + Z_{12}F_\mu(t)$. We consider $X(t)$ to be a gaussian random variable, i.e., that it represents the results of many small independent random events, in which case $Y(t)$ also is gaussian. That is, as a phase point moves in Vm space it is buffeted by the noise, but only the component pointing away from the separatrix is important. There is competition between the noise and the deterministic kinetics governing the evolution of the system. The probability of firing (excitation) is thus equal to the probability that $y_1(t) > 0$ as $t \to \infty$ (see Fig. 3). Because of the presence of noise, some phase points that initially start on the right-hand side of the separatrix end up at the resting singular point ("A" in the figure) and some that start on the left end at the phase point representing excitation. For a stimulus (short pulse of current) that rapidly moves the system to a phase point close to the separatrix, one finds[11] the following relationship between the probability of firing $P(\text{fire}|\Delta V)$ and ΔV,

$$P(\text{fire}|\Delta V) = \frac{1}{2}[1 + erf(\Delta V Z_{11}/V_1\sqrt{2}D^{1/2}], \tag{8}$$

where erf represents the error function, ΔV is the initial value of the voltage when measured relative to the point where the separatrix intersects the V axis, and D is linked to the noise sources according to

$$D = \int_0^\infty \int_0^\infty \exp(-p_1(\xi+\eta))\langle X(\xi)X(\eta)\rangle d\xi d\eta. \tag{9}$$

3. PHOSPHOINOSITIDE CYCLES CAN ACT AS TWO STATE SWITCHES FOR RECEPTOR MEDIATED ENDOCYTOSIS

Although the exact sequence by which the vesicles form is not clear, the basic cycle of clathrin coated vesicle physiology has been known for some time. Extracellular ligands (cargo) associate with transmembrane receptors, which bind adaptor proteins ("APs") from a soluble fraction in the cytoplasm. In turn, the APs bind clathrin triskelia that are adsorbed from the cytoplasm onto the membrane-bound receptor-adaptor complexes. It is known that APs have a high affinity for inositol phospholipids (phosphoinositides),[18] the latter being membrane lipids that contain inositol sugars bound to their acyl chains. Because the OH groups in the sugar rings can be specifically phosphorylated by selective kinases, the phosphoinositides can act as signaling molecules to bring about particular membrane events that enable various cell activities. Perhaps the most familiar of the phosphoinositides, because they participate in a large number of well-studied cell physiological events, are the phosphatidylinositol 4,5 bisphosphates ("PIP_2", or "$PI(4,5)P_2$"). Although present in lower concentrations, other important molecules are the 3' phosphoinositides $PI(3,4)P_2$ and $PI(3,4,5)P_3$ (the numbers signifying the locations on the inositol ring that are phosphorylated). Other than possibly being implicated in endocytosis, the 3' phosphoinositides play an important role in gradient sensing in eukaryotic chemotaxis.[19]

In addition to the APs, clathrin-binding proteins such as epsin which contain a domain known as an ENTH domain, also bind to the PIPs (notably $PI(4,5)P_2$) and, in vitro, impart curvature to membranes containing phosphoinositides.[20] Endophilin, which has been implicated in endocytosis *in vivo*, contains a banana-shaped moiety known as a "BAR domain" which bends membranes containing $PI(4,5)P_2$, though it may not bind directly to clathrin.[21] Also, because of their intrinsic curvature,[22] triskelions are likely to prefer to coalesce on rounded surfaces of certain types as they form closed cages, so recruitment of clathrin triskelia to a budding vesicle may be an intrinsically non-linear affair: ligand-bound receptor-AP complexes could concentrate and thereby attract clathrin molecules that polymerize into a partial basket which, because the bound clathrin recruits membrane-bending proteins, accelerates further binding of clathrin. The newly-bound clathrin then gathers free AP-receptor complexes diffusing in the plasma mebrane, which bind additional clathrin, which leads to more bending, etc. Details of such a scenario are not well known, and one purpose of mathematical modeling is to help define and guide experiments that will distinguish possible mechanisms.

Two additional functions are known: the binding of proteins, most particularly dynamin molecules that facilitate the scission of the vesicle from the plasma membrane,[23] and a mechanism usually ascribed to actin and other cytoskeletal elements that results in movement of the vesicles away from the plasma membrane towards the interior of the cell. Whereas the assembly of the coat by clathrin and its direct binding partners is believed to occur without nucleotide hydrolysis, the action of dynamin, and the displacement effected by cytoskeletal elements, clearly depends on molecular transformations brought about by transfer of energy stored in the phosphate groups of ATP or GTP. Another, somewhat less appreciated factor, is the likely involvement of monomeric GTPases in clathrin-coated vesicle biogenesis. The family of GTPases includes Rac, Ran, Arf, Arp, Akt, CDC42, etc., several of which are known to be involved in a number of membrane-proximal transformations at internal organelles as well as at the plasma membrane.[24,25] These relatively small proteins typically bind GTP which is hydrolyzed during cell reactions, after which the GTPase is recharged by replacement of GDP by GTP. Regulation of these transformations occurs through "guanine nucleotide exchange factors" ("GEFs") and "GTPase activating proteins" ("GAPs").[26] As illustrated in the cartoon in Fig. 4, this phenomenology is central to phosphoinositide metabolic cycles, which can couple binding events at the cell surface to the generation of phosphoinositides that bind factors that affect membrane curvature. Not only does this provide the necessary positive feedback for vesicle formation, but it also can give rise to a triggering mechanism that rationalizes the observation that coat proteins seem to coalesce at specific points on the cell surface. These cycles also can be coupled to other cellular factors and events, thereby lining receptor mediated endocytosis to other cell physiological functions.

It has been recognized for some time that clathrin, APs, and other endocytic proteins bind to $PI(4,5)P_2$ in vitro. Demonstrating the relevance of this observation in cells is still a subject of intense study.[27] The use of TIRF (total internal reflectance fluorescence) microscopy, which allows one to look at cellular events

occurring at the plasma membrane, coupled with advances in the production of visual fluorescence protein chimeras and newly-developed techniques to mediate phosphoinositide metabolism has led to the observation that formation of clathrin-coated pits is halted if cellular $PI(4,5)P_2$ is drastically depleted.[27] However, $PI(4,5)P_2$, when acted upon by the enzyme PI3 kinase, is a precursor for $PI(3,4,5)P_3$. Recent evidence indicates that $PI(3,4,5)P_3$ acts in concert with $PI(4,5)P_2$ to selectively bind small GTPases to the plasma membrane (in their polybasic, modified forms), and depletion of $PI(4,5)P_2$, alone, yet results in plasma membrane retention. The conclusion is that and $PI(4,5)P_2$ and $PI(3,4,5)P_3$ jointly regulate plasma membrane targeting of proteins having clustered polybasic residues.[28]

Vesicle formation is involved in other cellular trafficking processes, though the coat proteins generally are different. For example, the major coat proteins on vesicles effecting trafficking between the endoplasmic reticulum and the golgi are the COP proteins, COPI and COPII.[1] A good deal is known about the involvement of phosphoinositides and monomeric GTPases in these processes. Several years ago Randazzo and coworkers sketched a cycle of phosphoinositide metabolism believed to participate in vesicle biogenesis involving COPI at the endoplasmic reticulum.[29] Figure 4 is adapted from a figure in their paper, which contained the central figure (enclosed in the box) that illustrates how small monomeric GTPases are implicated in phospholipid transformations. Although the story has evolved over time, and the details of processes giving rise to other cellular membrane organelles (such as the plasma membrane) are somewhat different, some essential features are apparent: $PIP(4,5)P_2$ ($PtdInsP_2$) is converted to $PIP(3,4,5)P_3$ ($PtdInsP_3$) through the action of PI3 kinase (PtdIns3-kinase); $PIP(3,4,5)P_3$ regulates the activity of a guanine nucleotide exchange factor (GEF) that charges an ARF-family GTPase, which increases the conversion of a monophosphoinositide (PtdInsP) to $PI(4,5)P_2$; the level of $PI(4,5)P_2$ regulates the GTPase activating protein (GAP) to dephosphorylate the ARF protein and thereby decrease the rate of production of $PI(4,5)P_2$. The action of $PI(3,4,5)P_3$ on the GEF represents a positive feed forward loop in this scheme, while the $PI(4,5)P_2$ activity on the GAP (as well as the conversion of phosphatidyl choline PC to phosphatidic acid PA) represents negative feedback.

We have added additional feed forward elements in the form of the dashed lines pointing to the arrow signifying the action of PI3 kinase, and assume that membrane curvature enhances enzyme activity.[30, 31] An increase in membrane curvature might transiently occur as local charge is added to the phosphoinositide molecules during conversions involving phosphorylation, and we also recognize that clathrin lattice formation affects membrane shape as described in the previous paragraphs. These would act to accelerate vesicle formation. Countering this tendency is the negative feedback involving regulation of Arf Gap and, more indirectly, the decrement of free $PI(4,5)P_2$ due to the binding of APs and other clathrin-associated proteins, as well as hydrolysis of $PI(4,5)P_2$ leading to the production of diacyl glycerol and IP_3 . This process also needs a trigger, which might be achieved by PI3K activation that seems to occur when certain binding reactions take place between ligands and their specific tyrosine kinase or heterotrimeric G-protein coupled receptors.[32] Correspondence with the phenomenon of threshold fluctuations in nerves is completed by noting that once clathrin-coated basket formation commences, the reactions do not necessarily proceed to the point where complete baskets and vesicles form. Rather, some partially-formed structures seem to break up and vanish.[33, 34]

However complicated this might seem, by making some simplifications one can represent this system by equations that are mathematically tractable. For example, if one writes a series of chemical rate equations for the activation and interconversion of the various factors discussed above, one obtains a set accounting for the production and degradation of $PI(4,5)P_2$, $PI(3,4,5)P_3$, phosphatidic acid, etc., stimulation of ARF-GAP, PI3K, phospholipase D (PLD), etc., population of the GTP-bound activated form of ARF, equations to account for changes in curvature and a mechanism to couple the curvature changes into the phosphoinositide cycle, etc. These equations contain a plethora of rate constants, but by setting certain concentrations constant (over the time when a vesicle forms) and by grouping constants into "reduced coefficients," one can obtain a simple set linking $PI(4,5)P_2$ concentrations to dynamic phospholipase activity levels.[35] This 2-equation set demonstrates properties not unlike those of the Vm system. A range of variables exits where the solution space appears to exhibit two stable singular points and a saddle point, similar to the situation illustrated in Fig. 2. Other equation subsets lead to similar behaviors, potentially making this a rich field for mathematicians to mine.

4. DISCUSSION

We have introduced the notion that clathrin-coated vesicle biogenesis, although a complex phenomenon, is amenable to mathematical analysis that has interesting consequences. By judicious approximation, this process can be described by equations that show two-state behavior and capture the transition between subthreshold stimulation of vesicle formation and conditions leading to completed vesicles, incorporating mechanisms allowing phosphoinoisitide metabolic cycles to be coupled to membrane curvature to yield positive feedback loops that balance degradation of free $PI(4,5)P_2$. These equations can be simplified and manipulated by a procedure akin to that used to analyze threshold fluctuations in nerves, yielding expressions that link the probability of vesicle formation to characteristics of the stimulus and the fluctuating cellular environment. More extensive investigation may provide insights regarding the regulation of vesicle formation and thereby yield deeper understanding of how endocytic processes are regulated by environmental factors. Moreover, by introducing fluctuations in relevant variables and making use of observations of frustrated vesicle formation, one may better understand how seemingly unrelated cell activities are coupled to each other. One goal of the theory would be to provide a unifying predictive framework to test hypotheses and guide experimental design and quantitative assessment of data.

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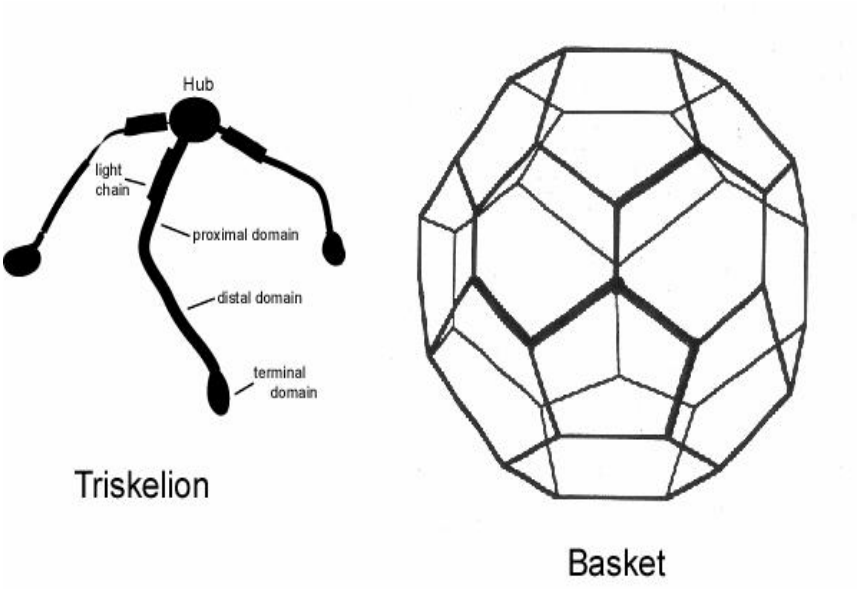

Figure 1. Cartoon showing clathrin triskelions and the polyhedral baskets into which they assemble. The legs of four triskelions form a strut in the polyhedron, each strut containing two proximal clathin leg segments and two distal segments.

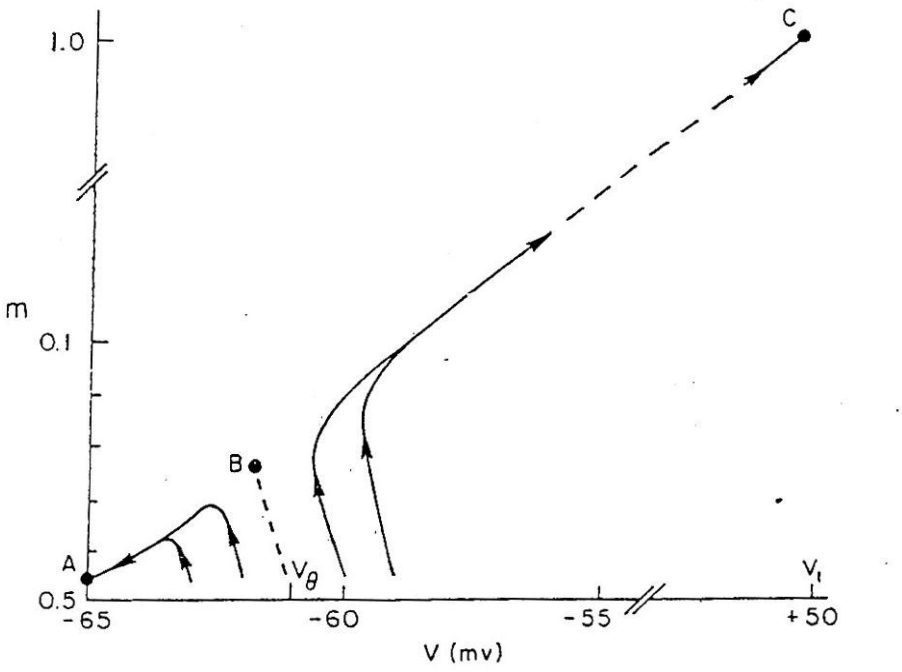

Figure 2. The (V, m) phase space, useful for assessing the early-time behavior of axonal response as described by the Hodgkin-Huxley (HH) equations. A and C are stable singular points, while B is a saddle point, herein identified as the "threshold singular point."

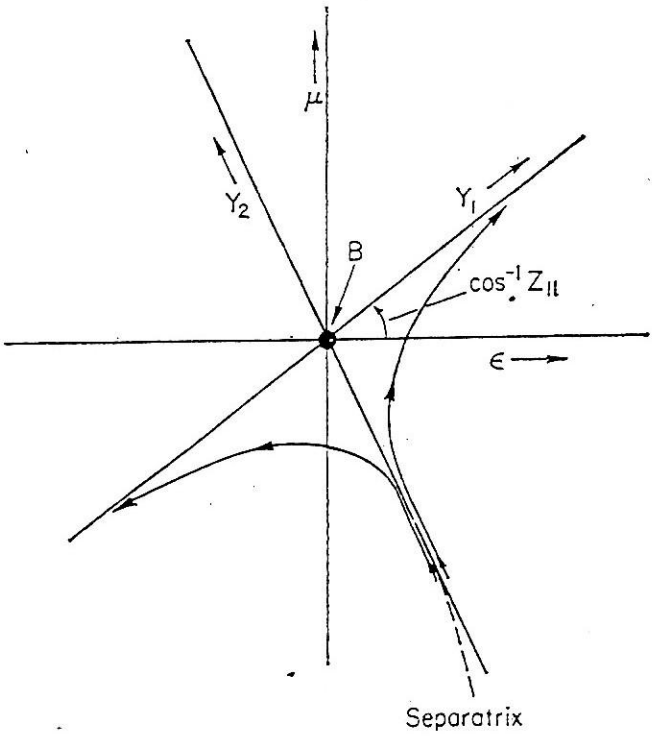

Figure 3. Linearized (V, σ) phase space, where σ is the Na conductance, related to the m variable of H-H as $\sigma = m^2$. In the vicinity of the threshold singular point (B) the kinetics associated with the non-linearity are preserved. Without noise a phase point always stays on the same side of the separatrix. Noise causes the phase point to undergo a random walk onto other trajectories. Depending on kinetic parameters, the strength of the noise and initial conditions, the system may end up at a stable singular point lying on the side of the separatrix opposite to that where it started.

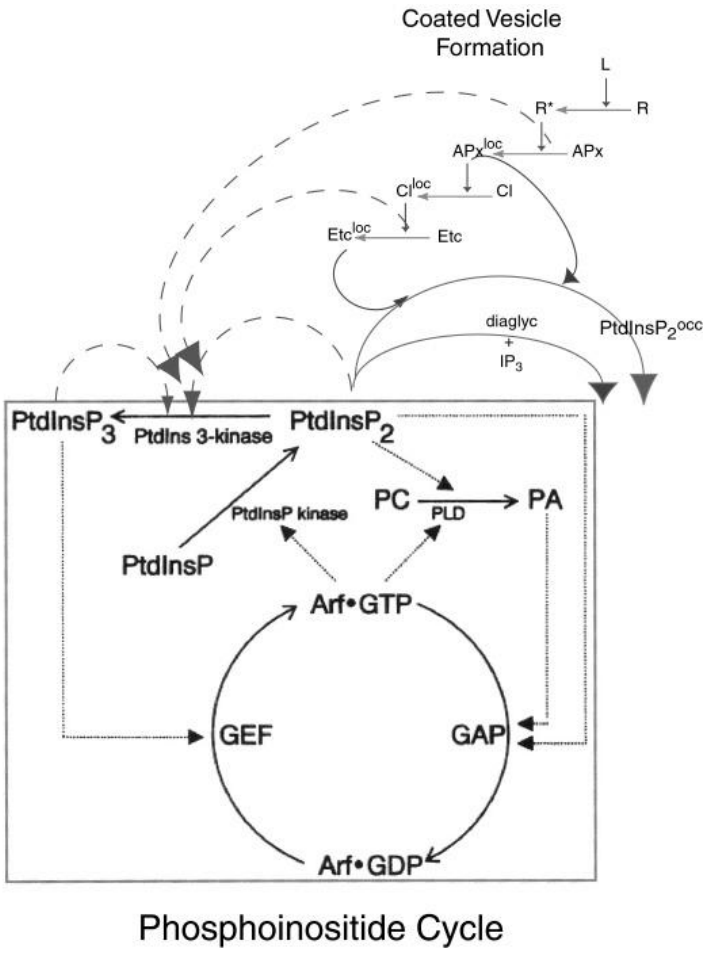

Figure 4. Example of a phosphoinositide control cycle,[29] showing competition between feed-forward and feedback elements (see text). Diagram has been modified to indicate how clathrin coat formation might be coupled into the metabolic cycle. (Note that, although ARF-family GTPases are implicated in the production of COPI-coated vesicles at the endoplasmic reticulum, different GTPases play a role in clathrin coated vesicle production at the plasma membrane.) This system might be triggered when PI3 kinase on the interior surface of the plasma membrane is activated by binding of ligands to transmembrane receptors.