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Open Cascades as Simple Solutions to Providing Ultrasensitivity and Adaptation in Cellular Signaling

Jeyaraman Srividhya^{1,2,*}, Yongfeng Li^{1,3,*}, and Joseph R. Pomeroy^{2,4,*}

¹Institute for Mathematics and its Applications, University of Minnesota, Minneapolis, MN, 55455, USA.

²The Biocomplexity Institute, Indiana University, Bloomington, IN, 47405, USA.

³USRA Division of Space Life Sciences, Houston, TX, 77058, USA.

⁴Department of Biology, Indiana University, Bloomington, IN, 47405, USA.

Abstract

Cell signaling is achieved predominantly by reversible phosphorylation-dephosphorylation reaction cascades. Up until now, circuits conferring adaptation have all required the presence of a cascade with some type of closed topology: negative-feedback loop with a buffering node, or incoherent feedforward loop with a proportioner node. In this paper—using Goldbeter and Koshland-type expressions—we propose a differential equation model to describe a generic, open signaling cascade that elicits an adaptation response. This is accomplished by coupling N phosphorylation-dephosphorylation cycles unidirectionally, without any explicit feedback loops. Using this model, we show that as the length of the cascade grows, the steady states of the downstream cycles reach a limiting value. In other words, our model indicates that there are a minimum number of cycles required to achieve a maximum in sensitivity and amplitude in the response of a signaling cascade. We also describe for the first time that the phenomenon of ultrasensitivity can be further subdivided into three sub-regimes, separated by sharp stimulus threshold values: OFF, OFF-ON-OFF, and ON. In the OFF-ON-OFF regime, an interesting property emerges. In the presence of a basal amount of activity, the temporal evolution of early cycles yields damped peak responses. On the other hand, the downstream cycles switch rapidly to a higher activity state for an extended period of time, prior to settling to an OFF state (OFF-ON-OFF). This response arises from the changing dynamics between a feed-forward activation module and dephosphorylation reactions. In conclusion, our model gives the new perspective that open signaling cascades embedded in complex biochemical circuits may possess the ability to show a switch-like adaptation response, without the need for any explicit feedback circuitry.

3 Introduction

Signal transduction is key to controlling major eukaryotic cellular processes including differentiation, cell cycle, metabolism, and programmed cell death. When a stimulating ligand (an effector) is recognized by its receptor, the message being transmitted for these processes is often conveyed by covalent modification of proteins. Effectors can stimulate pathways whose output can be either time-dependent or constant. The most common means of transmission operates via phosphorylation-dephosphorylation (PD) reactions, or *cycles*. The first cycle activates the next and is followed by subsequent cycles, thus propagating the signal until a response is elicited at the terminus of the pathway. A cycle could be composed

Address correspondence to Joseph R. Pomeroy (jpomeroy@indiana.edu).

*These authors contributed equally to this work

of one reversible PD reaction involving mono-phosphorylation of a kinase—a single PD cycle—while a dual PD cycle includes two reversible PD reactions involving double phosphorylation of a kinase. The Mitogen Activated Protein Kinase (MAPK) cascade is composed of a single PD cycle followed by two dual PD cycles (Figure 1A) [1]. Examples of single PD signal networks include class I Phosphoinositol-3-kinase (PI3K) pathways that are involved in processes like angiogenesis [2], apoptosis, and cell growth control [3]. In general, these signaling cascades can be “open” implying that a linear stretch of PD cycles that are coupled to each other without a feedback loop where signal passes unidirectionally through the pathway (Figure 1B). Alternatively, cascades can be “closed”, implying that the linear stretch of PD cycles are enclosed by an explicit feedback loop, and the resulting output feeds back through either a positive- or negative-feedback loop (Figure 1C). In this paper, for the sake of simplicity, we will refer to cascades with or without feedback loops as “closed” or “open”, respectively.

The fundamental structure of a generic open signal transduction cascade composed of single PD cycles is shown in Figure 1B. Here, Y and YP are the dephosphorylated (inactive) and phosphorylated (active) forms of the protein, respectively. For this generic cascade, several models [4–10] have been proposed using different forms of kinetics to describe the PD reactions. A well-known model for a single reversible PD reaction was proposed by Goldbeter and Koshland (GK) [11] and is based on Michaelis–Menten (saturation) kinetics. Following this model, experimental studies also revealed the presence of ultrasensitive phenomenon [1]. This model still serves as the basis of several mathematical models, including those of eukaryotic cell cycles [12–14], pharmacokinetics and pharmacodynamics [15, 16], and several other biochemical processes [17–20]. It also exhibits the special property called *zero-order ultrasensitivity*: when the stimulus intensity surpasses a certain threshold, the steady state levels of the kinase activities increase steeply and switch to even higher stable states. Therefore, the steady state concentrations of the active phospho-kinases show a steep sigmoidal response as a result of increasing stimulus strength (Figure 2A). The stimulus threshold at which this steep change of steady states occurs is unknown, as well as whether this threshold stimulus is a single value or an interval. However, it is known that this zero-order ultrasensitivity is enhanced as the number of PD cycles within a cascade increases. In fact, when four such PD cycles are coupled in an open cascade, Chock and Stadtman [21] showed that the overall amplification across the cascade increases by 10^{10} -fold. This observation raises an important question: is there a limit to the amount of amplification that could be achieved through the coupling of simple PD cycles?

Open signaling cascade models show transient temporal dynamics [4, 6, 9, 10] before reaching steady state. This was observed only when the saturation (Michaelis–Menten) kinetics in the GK model is approximated by first-order kinetics. This could occur in pathways that are weakly stimulated and the concentration of active kinases is very low when compared to total kinase levels. Under these conditions the GK model can be approximated to a first-order behavior. Heinrich et al. [4] proposed a model based on linear first-order kinetics for PD reactions, and the temporal response of the pathway resulted in peak-like signals before coming to rest at a steady state with a low concentration of active kinase. Chaves et al. [6] later extended this mechanism and identified optimal conditions for efficient signal transfer in cascades that are weakly stimulated. Qu and Vondriska [10] proposed a model for an open signaling cascade using a Hill-type function as an input from the previous cycle that also exhibited a transient temporal response. Most recently, Ventura et al. [9] proposed a model for this cascade that consisted of a series of PD cycles coupled such that the active form of the first cycle acts as an enzyme catalyzing the next PD cycle. Here, unlike the GK model, the model equations were derived through a standard singular perturbation analysis, with the kinetics of each PD cycle involving more than one variable. They discovered that the presence of an intrinsic feedback from each cycle to its predecessor

within the unidirectional pathway yielded oscillations. Markevich et al. [22] studied a linear cascade composed of dual PD cycles depicting the dynamics of Mos activation through MAPK cascade—this gave rise to bistability in the absence of an explicit feedback loop. Pomerening has described a detailed molecular mechanism for the behavior uncovered by Markevich et al. and referred to it as “intrinsic positive feedback” due to enzyme saturation, competitive inhibition, and feedforward inhibition [23]. Together, these various findings demonstrated that complex behaviors emerge from simple, open cascades, and suggested that additional rich dynamics could arise from this type of design. Despite their simple construction, open signaling cascades have the capability to show biological properties at steady state (ultrasensitivity) and within the dynamic regime (transient temporal responses). To date, behaviors like bistability (steady state) and adaptation (dynamic) have been shown to require biochemical circuitry that is essentially closed, through the implementation of one or more explicit feedback loops. The question remains: can an open signaling circuitry—without any overt feedback loop—provide these same types of outputs?

There are several examples of adaptation in biology [24–27], but there have been few modeling studies on this topic. Behar et al. performed a mathematical analysis of signal transduction pathways possessing feedback circuitry that can show adaptation behavior [28]. They considered seven different kinds of models that can exhibit minimal to strong adaptation behavior. Ma et al. found that there are two motifs in biochemical circuits that can provide a robust adaptation response [29]. Each motif consists of at least three nodes, with each node representing a PD cycle. They have identified that circuits conferring adaptation fall into two categories: negative–feedback loop with a buffering node, and incoherent feedforward loop with a proportioner node. What is common between both studies is that the pathways are closed and consist of some type of explicit feedback. Despite the fact that a cascade of PD cycles increases sensitivity and amplification, there is no evidence that this type of design can yield adaptation. However, open signaling systems may very well have provided a simple initial solution to adaptation, as well as other types of biological response. With this, we hypothesize that open signaling systems may very well have served as the precursors to the more robust, closed-feedback–driven systems that are prevalent today. We hereby present a mathematical model to show for the first time that an adaptation response can be achieved by “feedback-free” open signaling cascades. The most significant aspect of our model is that it is devoid of any kind of explicit positive- or negative-feedback loop, and yet it can extend the period of time that the output is in the ON–state prior to its settling to the OFF–state. Our model also answers the following questions regarding ultrasensitive phenomenon that have not been addressed by other open cascade models: (i) is there a limit to the amplification provided by an ultrasensitive and open multistep PD cascade? (ii) if so, what is the minimum number of cycles required to reach this amplification, and (iii) is there an exact threshold stimulus at which the ultrasensitive transition is defined?

Our model is composed of coupled ordinary differential equations describing an open signaling cascade constructed with GK–type expressions. As a general case, we couple N single PD cycles in a series. We show that when single PD cycles are coupled in an open cascade, the steady states of the downstream cycles reach a limiting value as the length of the cascade grows. Consequently, we find that under homogeneous parametric conditions, there is a limit to the amplification that can be provided by the cascade. We found that there is a clearly defined threshold stimulus value where the domain of ultrasensitivity begins, and that this threshold can be determined explicitly. To date, no other open signaling cascade models have exhibited this characteristic at steady state, nor have they shown an adaptation response in the temporal regime. What is most surprising is that an open signaling cascade can give rise to such complex behaviors. Here, we present the mathematical basis for these

rich dynamics, and describe a possible molecular mechanism in which they could be elicited biologically from simple open signaling systems.

4 Model

A simple and generic representation of an open signaling cascade is shown in Figure 1B. This schematic shows a receptor receiving a signal, which then transmits this signal by covalently modifying the protein Y_1 . Protein Y_1 is phosphorylated into a modified form, YP_1 , which is catalyzed by an enzyme kinase, E_1 . Simultaneously, YP_1 is dephosphorylated by the enzyme, E_1' . The modified or active protein (YP_1) then stimulates the next cycle. Therefore, each hypothetical PD cycle is linked to the next through a forward activation. The final cycle transmits the signal, which is then transformed into an output. In biological systems, the modified protein is often a phosphorylated kinase [1]. In order to maintain generality in the model, YP and Y are active and inactive kinases, respectively, and the enzymes that catalyze these phosphorylations are also "kinases". In addition, each cycle and its enzymes are assumed to be independent of the rest of the cycles. For simplicity, we represent YP as X in the equations. The ordinary differential equations describing the model are as follows.

$$\frac{dX_i}{dt} = V_i \frac{(1-X_i)}{K_i + (1-X_i)} - V_{Mi} \frac{X_i}{K_i' + X_i}, \quad (i=1, 2, 3, \dots, N) \quad (1)$$

where

$$V_i = \begin{cases} V_{Mi}; & i=1 \\ V_{Mi} [1 + \alpha_i X_{i-1}]; & i=2, 3, \dots, N \end{cases} \quad (2)$$

In Equation 1, conversion of X_i to Y_i is catalyzed by a kinase (E_i) and the reverse reaction by a phosphatase (E_i'). X_i and Y_i are scaled with total protein concentration, C_i , and hence, mole fractions of the active and inactive forms of the protein, respectively. Here we apply an important constraint [11] for observing the zero-order behavior in the Michaelis–Menten kinetics: the amount of the enzymes (E_i and E_i') catalyzing the PD reactions are very small when compared to the total concentration of the substrate and product (active and inactive, respectively). Thus, the fractions of the enzyme substrate complexes of X_i and Y_i are negligible such that the total fraction of the protein is simply $X_i + Y_i = 1$. Hence, the constraint $E_{iT} \ll 1$ is applied in the derivation of the rate functions. The parameters

E_{iT} , $E_{i'T}$, K_i , K_i' are also scaled using C_i . The parameter $V_{Mi} = k_i E_{iT}$, where E_{iT} is the total amount of kinase (for a detailed derivation please refer [11]). Since each PD cycle activates the subsequent cycle, the activation is mathematically represented using a linear function represented in Equation 2, where α_i defines the strength of activation. When a stimulus is applied via an effector, it affects the cascade by altering the total kinase concentration. Therefore, the total kinase concentrations are represented by the maximum rate parameter $V_{Mi} = k_i E_{iT}$. This is similar to the stimulation through E_{1tot} in the work of Huang and Ferrell [1]. We also assume a constant value for V_{Mi} , implying that the pathway is permanently stimulated. Explanation of the other parameters are given in Table 1.

It is important to note that in our model each phosphorylation and dephosphorylation reaction set is catalyzed by an independent kinase, E_i , and phosphatase, E_i' , respectively. Therefore, the active form of the i -th cycle, X_i , is not the kinase for the $i+1$ th cycle, as

described previously in the model by Ventura et al. [9]. Hence, the assumption that $E_{iT} \ll 1$ leads to $X_i + Y_i = 1$, and thus can be considered valid for each of the cycle.

5 Results

5.1 The steady states of downstream cycles converge to a limiting value

The model described above exhibits the property of ultrasensitivity, in that the steady states show a sigmoidal response as V_{Mi} is increased. It is also monostable under homogeneous parametric conditions and asymptotically stable (Supplementary Material S1). We consider $V_{Mi} = V_M$, $K_i = K_i' = K$ and $\alpha_i = \alpha$ for all simulations and analyses unless otherwise mentioned. The analytical expression to estimate the steady state of cycle 1 has been given by the GK model for a single reversible PD reaction [11]. The estimation of the rest of the steady states can be done using a recursive relation (Supplementary Material S2). The steady state response for the open cascade model described above for $i=1,2,\dots,7$ reveals that the steepness of transition increases as i increases (Figure 2B). An interesting property of the response emerges as we move towards the downstream cycles. For a given value of stimulus, V_M , as the length of cascade grows to infinity, the final steady state of the downstream cycles (large i) reaches a limiting value: X_{∞}^* . This can be represented mathematically as follows:

$$\lim_{N \rightarrow \infty} X_N^* = X_{\infty}^* \quad (3)$$

The mathematical proof of this is given in Supplementary material S3: Theorem 10.1. The limit in the model arises primarily due to the assumption in the GK model [11] that the total concentration of the phosphorylated and dephosphorylated forms of protein are constant; after normalizing them we obtain $X_i + Y_i = 1$. This condition imposes a mathematical boundedness in the equations. Also, this assumption has been followed in many mathematical models describing cell cycle and PD cascades, for example: Novak et al. [13, 30], Gonze and Goldbeter [31], and Goldbeter and Koshland [11]. Therefore, we adopted this assumption from the paper by Goldbeter and Koshland [11]. A further detailed explanation for the existence of this limit is described in prior work by Jeyaraman and Li [32].

5.2 The steady-state limit leads to a reduced model with bistability

From the GK model, it is difficult to discern the exact stimulus value at which ultrasensitivity originates (Figure 2A). Nevertheless, our model reveals that the domain of ultrasensitivity begins at a single sharp threshold (Figure 2B). We can therefore broadly subdivide this steady state diagram into *pre-ultrasensitive* and *post-ultrasensitive* regimes separated by this threshold stimulus value. Mathematically, this threshold can be determined by applying the limit (Equation 3) to the steady state equations of the model. First, we apply steady-state conditions to the ODE system by setting the rates to zero.

$$\begin{aligned} 0 &= V_M \frac{(1-X_1^*)}{K+(1-X_1^*)} - V_M' \frac{X_1^*}{K'+X_1^*} \\ &\vdots \quad \quad \quad \end{aligned} \quad (4)$$

$$0 = V_M (1 + \alpha X_{N-1}^*) \frac{(1 - X_N^*)}{K + (1 - X_N^*)} - V_M' \frac{X_N^*}{K' + X_N^*} \quad (5)$$

According to Equation (3) from Section 5.1,

$$\lim_{N \rightarrow \infty} X_N^* = X_\infty^* \quad (6)$$

When we apply this limit to Equation 5, ignoring the first cycle we get

$$0 = V_M(1 + \alpha X_\infty^*) \frac{(1 - X_\infty^*)}{K + (1 - X_\infty^*)} - V_M' \frac{X_\infty^*}{K' + X_\infty^*} \quad (7)$$

Rearranging Equation 7, we get a cubic polynomial $g(X_\infty^*)$ in X_∞^* such that $g(X_\infty^*) = 0$ and

$$g(X_\infty^*) = X_\infty^{*3} + X_\infty^{*2} \left[K' - 1 - \frac{1}{\alpha} \left(\frac{V_M'}{V_M} - 1 \right) \right] - X_\infty^* \frac{1}{\alpha} \left[\frac{V_M'}{V_M} (K + 1) - K'(\alpha - 1) - 1 \right] - \frac{K'}{\alpha} \quad (8)$$

For certain fixed α and $K = K'$ (say $\alpha = 1$ and $K = 0.01$ as given in the simulations), the limiting steady state X_∞^* depends on the stimulus parameter, V_M , in different ways. When we solve the cubic polynomial above, we obtain three real positive roots. However, three real positive roots are not observed for all values of stimulus parameter, V_M . Precisely, there exist two values, V_1^* and V_2^* , such that Equation 8 has only one positive root as $V_M \in (0, V_1^*) \cup (V_2^*, \infty)$ and three positive roots when $V_M \in (V_1^*, V_2^*)$. Here, the threshold value, V_2^* , is the actual threshold of ultrasensitivity.

Remark 1 Indeed, for some different sets of values for α and K , the prescribed V_1^* and V_2^* may not exist. In this case, Equation 8 always has only one root.

On the other hand, as N increases to be sufficiently large, the steady states X_N^* and X_{N-1}^* are very close. Therefore, after a sufficiently long time, the input from the preceding cycle $X_{N-1}(t)$ to the N th cycle will be very close to response $X_N(t)$ in this cycle. In this sense, the limiting behavior of $X_N(t)$, as $t \rightarrow \infty$, can be captured by the following reduced system

$$\frac{dX}{dt} = f(X, X) = V_M(1 + \alpha X) \frac{(1 - X)}{K + (1 - X)} - V_M' \frac{X}{K' + X} \quad (9)$$

which is a single reaction cycle embedded in its own positive-feedback loop. We describe the above equation as the *reduced model* and the Equation 1 as the *primary model*.

Note that the reduced model (Equation 9) has exactly three equilibrium points, $\beta_1^*, \beta_2^*, \beta_3^*$, as $V_M \in (V_1^*, V_2^*)$ (see Figure 2C where $V_M = 0.75$); here, β_1^* and β_3^* are stable, while β_2^* is unstable. For $V_M > V_2^*$ or $V_M < V_1^*$, Equation 9 has only one equilibrium point which is hence stable. This reveals the presence of bistability in reduced Equation 9. Therefore, the threshold stimuli V_1^* and V_2^* form a range (V_1^*, V_2^*) beyond which bistability ceases to exist in the reduced model. A detailed mathematical analysis of this model has been described previously Li and Srividhya [32].

5.3 The reduced model defines three sub-regimes of ultrasensitivity

The parameter ranges of the reduced model described above dictate certain characteristics in the primary model. It is only by reducing the primary model to the reduced model that we can determine the stimulus parameter, V_2^* , which is precisely where the ultrasensitive switch

originates. Based on the bistability parameter ranges of the reduced model (Equation 9), we can sub-divide the steady state diagram of the primary model (Figure 2B) into three regimes (Figure 2D). The regimes:

- Regime I (one real positive root β_1^*) $V_M < V_1^*$
- Regime II (three real positive roots $\beta_1^*, \beta_3^*, \beta_3^*$) $V_1^* < V_M < V_2^*$
- Regime III (one real positive root β_3^*) $V_M > V_2^*$

In Figure 2D, the steady states of both the primary and reduced models are shown together, where the bistable regime of the latter is depicted (gray and blue circles), as well as the ultrasensitive curve of the primary model, for a cascade $N=7$ (black line). In the primary model, regimes I and II are classified as *pre-ultrasensitive* and the regime III as

ultrasensitive, respectively. In biological terms, if the stimulus is greater than the value V_2^* , the steady-state activities of the downstream cycles will switch to a permanent ON state.

5.4 Peak responses arise during the temporal evolution of the primary model

In the pre-ultrasensitive regime, the time evolution of the concentrations of the active proteins in the cascade will always reach an OFF state. However, the existence of a steady-state limit, as well as different regimes, may be indicative of surprising dynamics in the time evolution of the model. Therefore, we numerically simulated the system of ODEs shown in Equation 1, where we assumed an open cascade composed of N PD cycles. The phosphorylated active forms are indicated as X_i , where i represents the index of the downstream cycles. For example, X_1 indicates the first cycle, X_2 represents the second cycle, and so on. We also assign initial values for all of the active forms X_i^0 in each cycle. As mentioned in Section 4, the concentrations of the active and inactive form of the protein are represented as fractions.

Biologically, it is conceivable that each PD cycle is not exclusive to one cascade and may be connected to several other networks of cascades. It is therefore possible that there may be one or more inputs to a given PD cycle, such that the initial or the basal activity of one cycle can be higher than that of the downstream cycles. Thus, in this case we will make the assumption that one of the cycles is activated through some arbitrary mechanism. This can be represented as an initial fraction greater than the steady state, such that $X_1^0 > X_1^*$ and $X_{2,...,N}^0 = 0$. For instance, an initial fraction of $X_1^0 = 0.3$ for a PD cycle would imply that the cycle has 30% of its total protein concentration in the active form. Starting from this initial condition, a transient peak response was observed, meaning that there was an initial increase in the concentration of active protein over time, followed by a decrease (Figure 3). In all, there were three different temporal behaviors observed:

- *Regime I or OFF regime* - $V_M < V_1^*$: In this region, for $X_1^0 > X_1^*$ and $X_{2,...,N}^0 = 0$, we observe negligible or no peak response (data not shown). Irrespective of the X_1^0 , the cascade always yielded a damped peak response, and eventually settled to a stable OFF state.
- *Regime II or OFF-ON-OFF regime* - $V_M \in (V_1^*, V_2^*)$: For $X_1^0 > X_1^*$, we see two types of peak responses, depending upon the fraction of the initially active form that is present in the first cycle. For $X_1^0 = 0.25$ (25% active form), as the number of PD cycles increases, we see slightly increasing levels in peak response that damp prior to reaching a low steady state (Figure 3A). On the other hand, when the initial fraction of the active form of the first cycle is increased to $X_1^0 = 0.35$ (35% active

form), we observe increasing peak responses as the cycle number increases, along with sustained activities past a sufficient number of downstream cycles (Figure 3B). For both simulations the other parameters were held constant ($V_M = 0.815$, $\alpha = 1.55$, $K = 0.008$), revealing that increasing the initial fraction alone can turn a damped signal into an amplified signal. Likewise, this behavior can be replicated by fixing the initial fraction and changing the parameter $V_M \in (V_1^*, V_2^*)$ (data not shown). This effect is similar to what is described in the linear cascade model of Heinrich *et al.* [4], where amplification is observed for higher values of the ratio of kinase-to-phosphatase activity rates. Along with the downstream activity amplification in our model, the maximum amplitude of cycles $N=5,6,7$ reaches an identical maximum limiting value X_{max} (Figure 3B). These cycles reach this X_{max} in an abrupt, switch-like fashion rather than being activated gradually, and then remain at X_{max} for increasingly prolonged periods as N increases. As this model evolves over time, these downstream cycles yield an output reminiscent of a square wave. As long as $V_M < V_2^*$, the final steady state responses always settle to the OFF state. Thus, we refer to the response of the model in this regime as an "OFF-ON-OFF" type. The output of the model for $N=7$ is a typical adaptation response, where the concentration of the active form increases and then returns back to the inactive state while still under the influence of a constant stimulus (Figure 3B, inset). Also we see that the X_{max} and X_7 conform with the β_3^* and β_1^* of the reduced model. β_2^* is the unstable steady state in the hysteresis curve of the reduced model, and therefore is theoretically impossible to reach during the temporal evolution of the model. When measured experimentally, this response is practically indistinguishable from an "all-or-none" response, and could be indicative of a reversible bistable steady-state response ([33], Figure 2A). Although this could be deemed a transient temporal response to a constant stimulus, the transition to the higher activity state is abrupt and switch-like. The model proposed by Qu and Vondriska [10] also shows a similar result, with the signal strength reaching the same magnitude as the cascade length increases, regardless of the stimulus applied. Their model uses Hill-type equations with an exponent to describe the reaction kinetics.

- *Regime III or ON regime* - $V_M > V_2^*$: Irrespective of the X_1^0 values, the output from the downstream cycles always reaches the ON state. For $X_1^0 < X_1^*$, ON states are reached monotonically (Figure 3C); if $X_1^0 > X_1^*$, then the ON states arise through a transient peak response, although the final steady-state activities may be reduced (Figure 3D).

The significance of the steady-state limit (Equation 3) arises when it is applied to the steady-state equations (Equations 4 through 5). This in turn permits us to separate the three regimes through Equations 8 and 9. This permitted identification of the stimulus range that can give rise to "OFF-ON-OFF"—an adaptation response—during the time course. We believe this is the major advantage of discovering the mathematical limit (Equation 3), while biologically, it is not possible to compute this limit. The linear cascade model has a unique steady state, and as long as this system is in the pre-ultrasensitive regime, it will always settle to the stable OFF state. However, if the system is in the OFF-ON-OFF regime, it will exhibit a switch-like temporal response before settling to the OFF state.

5.5 The steady-state limit (X_∞^*) and the maximum concentration limit (X_{max}) of the primary model

It is important to note, that mathematically, an infinite number of cycles are required to achieve the limit X_∞^* at the final steady state. Depending on the parameters, only a few

cycles are required to achieve the maximum limit, X_{max} , during the time course. Therefore, there are two limits: (a) X_{∞}^* : the final steady-state limit, and (b) X_{max} : the maximum concentration limit in the time course. We also find that as the cascade length increases, the X_{max} and X_i^* becomes closer to the roots of the reduced bistable model β_3^* and β_1^* , respectively – this is also indicated in Figure 3C. Furthermore, it is important to note that reaching X_{max} is an essential condition of the OFF-ON-OFF response. When a given cycle reaches the limit X_{max} during the time course, then the subsequent cycles also reach the same value and exhibit this OFF-ON-OFF response.

5.6 Mechanisms that yield the OFF-ON-OFF behavioral regime

We have postulated a plausible molecular mechanism that can yield the previously described OFF-ON-OFF response of our model (Figure 3B). For clarity, throughout the following section we will refer to the phosphorylated form as, “YP”, rather than “X”.

5.6.1 OFF to ON—If a cascade of PD cycles is initiated by an effector, and there is a sufficient basal concentration of YP_1 (the first active protein), the rapid conversion of Y_2 to YP_2 begins. Here, we assume that the conversion of Y_2 to YP_2 by the enzyme E_2 occurs, (a) without the aid of YP_1 , and (b) with the aid of YP_1 . In terms of molecular mechanism, YP_1 can be envisioned attaching itself to Y_2 to form another intermediate species, Z_2 . Starting in the OFF state, the forward- and back-reactions of Y_2 phosphorylation and YP_2

dephosphorylation (via E_2 and E_2' , respectively) remain balanced (Figure 4A). Therefore, Y_2 predominates at this stage (Figure 4B). Eventually a basal level of YP_1 accumulates, and the formation of Z_2 subsequently enhances the conversion of Y_2 to YP_2 in the module as the phosphorylation rate of the former exceeds the dephosphorylation rate of the latter (Figure 4C, D). Although we have not invoked the variable Z_i in our model, we have represented it here in order to describe a plausible feed-forward module. This is similar to a “coherent feed-forward” step proposed by Mangan and Alon [34]. This feed-forward step is rapidly amplified and propagated downstream producing additional YP_i s. It must be noted that only in the presence of a certain basal amount of YP_1 is the feed-forward step amplified downstream. If the basal amount of YP_1 is too low, the feed-forward step is damped and no further dynamics occur.

In addition to the feed-forward module, there is another regulatory mechanism in place, namely the dephosphorylation reactions driven by phosphatases. Due to the feed-forward amplification, an excess of YP_2 is produced and effectively saturates the phosphatase E_2' . Therefore, dephosphorylation is essentially inhibited via phosphatase saturation and gives rise to an overall inhibitory effect upon E_2' (Figure 4C; note YP_2 inactivating E_2'). As a result of this saturation, even small amounts of Y_2 are converted immediately into YP_2 (Figure 4D). Due to this feed-forward amplification and saturation inhibition there is a massive flux of phosphorylation, and hence, all downstream cycles become active and switch rapidly from the OFF to the ON state.

5.6.2 Temporary ON—Once all of the Y_i s are converted into YP_i s, the ON state is maintained temporarily (Figure 4E, F). This occurs due to the balance that is reached between the feed-forward and dephosphorylation (E_2') reaction rates.

5.6.3 ON-OFF—The feed-forward amplification that occurs in the model eventually subsides because there is not enough basal YP_1 to sustain the chain reaction occurring in subsequent cycles - this means that the ON state is only temporary. The initial basal kinase activity in the first cycle is depleted, and hence, there is not enough YP_1 to support Y_2 -to- YP_2 production (Figure 4G). This inactivating effect then propagates and breaks the feed-

forward loop. At this point only the phosphorylation and dephosphorylation reactions—without the feed-forward loops—remain, and the system returns from the temporary ON state to the OFF state (Figure 4G, H). It is critical to note that the switching of the output from OFF to ON is much more rapid than ON to OFF. This clearly shows that the OFF to ON transition is aided by two mechanisms: namely feed-forward amplification and saturation inhibition. The ON to OFF transition, on the other hand, is governed by phosphorylation-dephosphorylation reactions alone.

5.7 Effect of the fraction of initial active protein on OFF-ON-OFF response

In an actual biological scenario, when a pathway is connected to other pathways, it may be realistic to have inputs that can elevate the basal activity of a specific cycle. This raises the question of how much initially active protein—with respect to the total concentration—is required to achieve the aforementioned OFF-ON-OFF response? In the reduced model that exhibits bistability (see Equation 9), the threshold for the ON state is the unstable equilibrium point, β_2^* . If the initial fraction is greater than β_2^* , then the reduced model flips to the ON state. However, this is not true for the primary model (see Equation 1). $X_1^0 > \beta_2^*$ is a necessary condition but it is not sufficient to flip the activity of the system to the temporary ON state. X_1^0 should be above a certain threshold, $\bar{X}_1$, such that $X_1^0 > \bar{X}_1 > \beta_2^*$ to reach the ON state during the time course. Due to the highly non-linear nature of the equations describing the system, this threshold initial fraction, $\bar{X}_1$, cannot be determined analytically. Therefore, we have conducted numerical simulations to obtain a reasonable measure of $\bar{X}_1$. From our model, the maximum activity of the downstream cycles 5, 6, and 7 reaches X_{max} , whereas cycles 1, 2, 3, and 4 show smooth peaks and do not reach this value (Figure 3B). In order to generalize, we call the maximum activity reached by any given cycle during the time course as X_i^m . For small N , $X_i^m < X_{max}$ (generating a smooth peak), and for larger N , $X_i^m = X_{max}$ (generating a square wave). In order to obtain an approximate measure of the threshold $\bar{X}_1$, we numerically estimated the X_i^m with respect to initial fraction X_1^0 , for a coupled cascade with 7 PD cycles (Figure 5A). When the initial fraction X_1^0 (the percentage of initial active protein) is increased, the X_i^m increases smoothly for cycle 2. For cycle 7, the X_i^m remains low and jumps abruptly to X_{max} at $X_1^0 = 0.3$ (30% of the total protein concentration). Within the OFF-ON-OFF regime, this is the threshold $\bar{X}_1$ for amplifying the signal downstream, as well as to flip the system to a temporary ON state. Another observation is that the early cycles show a graded increase in their peak amplitudes, whereas the downstream cycles show an "all-or-none" response with respect to increasing X_1^0 .

Furthermore, we numerically derived the $\bar{X}_1$ required to observe the OFF-ON-OFF response with respect to increasing the stimulus parameter, V_M (Figure 5B). As the stimulus increases, the amount of initial active protein required for an OFF-ON-OFF response decreases linearly and would therefore permit the system to flip more easily. For stimuli close to the ultrasensitive threshold, OFF-ON-OFF behavior is observed from cascades with 3 cycles ($N=3$) (Figure 5C). Together, these results show that an OFF-ON-OFF output from a signaling pathway requires only a short cascade with low initial activity, and suggests that this simple recurring topology could serve more complex functions in biological systems than first thought.

5.8 Effect of parameters on the three regimes

The range of the three regimes is dependent upon all of the parameters of the model— V_M , α , and K (Figure 6)—and this can be derived from the reduced model (Equation 9). As shown in a two-parameter diagram ($V_M - \alpha$), the range of the OFF-ON-OFF regime widens as the

value of α increases (Figure 6A). As K increases ($V_M - K$), however, the OFF-ON-OFF regime narrows; for $K=0.085$, the regimes cease to exist altogether (Figure 6B). It is known that as K increases, the ultrasensitive behavior is lost and the system enters a first-order behavior [11]. Therefore, it is apparent that the PD cycles within an open cascade must be tightly coupled (i.e. strongly activate each other), and that the system would require a low Michaelis–Menten constant to yield the OFF-ON-OFF behavior.

6 Discussion

We have presented here a mathematical model of an open signaling cascade, comprising single PD cycles, using well-known Goldbeter-Koshland (GK) expressions. We coupled N - PD cycles through a forward activation, and described the dynamics using coupled ordinary differential equations. By merely coupling PD cycles, our model can produce a highly efficient adaptation response in the absence of any feedback circuitry. First, we showed that a GK-type open signal cascade can exhibit peak signals, provided the initial concentration of the first, or one of any intermediate kinases in the cascade has a basal level of activity. Under this condition, when the length of the cascade increases, the steady-state concentrations of the active kinases of the downstream cycles reach a limiting value. Consequently, we reduced the N -variable primary model into a one-variable reduced model that exhibited classical bistability. Using the bistable regimes in the steady-state diagram of the reduced model, we sub-divided the ultrasensitive phenomenon of the primary model into three distinct regimes. We discovered that one regime existed in particular—OFF-ON-OFF—that elicits a classic adaptation response in the absence of any overt feedback loop. Lastly, we propose a molecular mechanism that explains how this dynamical behavior could occur during a time course.

Our model represents a simple open cascade of PD cycles, showing a well-known ultrasensitive response in the steady state. However, in such a steady-state response curve, the exact value of the threshold stimulus after which the ultrasensitive switch originates is not obvious (Figure 2B). It is only through the steady-state limiting value that we have discovered that we can identify this threshold stimulus. Discovering this threshold may help in the engineering of pathways in order to produce specific outputs.

Additionally, with the help of the reduced model—obtained by applying the steady-state limit to the primary model—we were able to distinguish the various regimes in the steady-state diagram (Figure 2D). The primary model, in the meanwhile, shows intriguing time evolution behaviors within these regimes. Particularly in Regime II, we observe increasing peak responses in the activity of downstream cycles, before settling to the OFF state (Figures 4A and 4B). Notably, when the initial fraction is above a certain threshold, we observe a time course that is reminiscent of a square wave (Figure 4B, inset). This is, as far as we know, the first model that highlights such a behavior in the output of downstream cycles in a feedback-loop-free cascade. The square wave suggests that the cascade is capable of residing in a temporary ON state for prolonged periods, and then falling back to the OFF state, and we have postulated a plausible molecular mechanism that can bring about this behavior. The most important aspect of our proposed mechanism involves the presence of an intrinsic feed-forward step. The combination of a feed-forward step with phosphatase inhibition brings about the switching of the activities of the proteins in the cascade from OFF to ON during the time course. The temporary ON state is maintained by the balance in the rates of the feed-forward step and dephosphorylation, while the transition from this temporary ON state to OFF is mediated by phosphorylation–dephosphorylation reactions alone (Figures 3A–H).

Behaviors such as those described above are not unfamiliar to cellular signaling systems. For example, Markevich *et al.* have developed a quantitative model for Ras activation [35]. In their experiments—despite maintaining constant stimulus levels (EGF)—they observed transient peak activities in the proteins involved, before the system settled to a steady state (Figures 4, 5, 7, 9 in [35] and Box 1 in [36]). The explanation for this behavior was that activation of Ras occurred through mechanisms independent of the pathway, and that Ras is a point of both convergence and divergence of multiple signaling pathways [35]. An important property shown by our model is the transient peak response in the ON regime, due to an elevated initial concentration of the active kinase of the first PD cycle. This elevated initial concentration can be conceived as a consequence of independent activation mechanisms, as well as connections with other pathways. The time evolution of our model in the ON regime shows transient behavior in the response of downstream pathways before they settle into their respective steady states (Figure 4D). Therefore, our model supports the hypothesis that transient peak responses within a pathway can occur due to activation mechanisms that are independent of that pathway.

In our model, cycles most upstream ($N = 2$) in the cascade show a graded response (red), whereas downstream cycles ($N = 7$) show a switch-like or digital response (blue) with respect to change in stimulus and initial fraction (Figure 5A). Indeed, this has been observed in experiments where a graded response precedes a switch-like response in *Xenopus laevis* oocytes [37]. This supports the notion that length of the cascade—and particularly, the cycles further downstream—may play the major role in converting the response from analog to digital. What is unique about our discovery is that the coupling of PD cycles without any extrinsic feedback loops has the potential to yield an all-or-none response and temporarily sustain that output under continued stimulation, reminiscent of an adaptation response. Adaptation is a well recognized biological phenomenon, but there exist very few mathematical models describing this process. The modeling study conducted by Behar *et al.* consisted of several types of circuits involving PD reactions that conferred an adaptation property [28]. Their circuits are closed and require a feedback regulation (negative-feedback or inhibition) in order to exhibit adaptation. In their model, the stimulus is constant over time and applied at the start of simulation (at $t = 0$). Their analysis led to the conclusion that (a) multistep cascades improve sensitivity, as well as signal amplification; (b) some feedback architectures act as signal transducers that convert signal strength into response duration; and (c) feedback deactivation acts as a dose-dependent switch between transient and sustained responses.

Recently, Ma et al. [29] showed that robust biochemical adaptation can be achieved only by two major core topology or circuits: either a negative-feedback loop with a buffering node, or an incoherent feedforward loop—which implicitly contains an inhibitory leg—with a proportioner node. The most important aspect of their finding is that these two different topologies require at least three PD cycles connected in a closed circuit. In their model, a constant low stimulus is introduced and the circuit is evolved to steady state. The stimulus is then increased to a higher value. Along with this adaptation model, there exist a few others involving different modes of regulation, including enzymatic, proteolytic, transcriptional, and protein-protein interaction [38–40]. According to Ma et al., all of these models fall under the two topologies described above [29]. From these prior studies, it is clear that there is still no standard way of simulating adaptation.

Our model has the following features that distinguish from the aforementioned models: (i) it is a multistep open cascade of PD cycles; (ii) it does not require an overt negative-feedback loop (or positive feedback) to yield adaptation; and (iii) it applies a constant stimulus from the start of simulation ($t = 0$). Combining these features, we have shown for the first time that an open signaling cascade can yield adaptation, and that this behavior can arise free of

any type of feedback loop. To yield an adaptation response, our model does have certain requirements. First, the simulation of our model requires an elevated initial concentration of first or any intermediate PD cycle in the cascade in order to yield a signal peak behavior, whereas other adaptation models do not require this condition. This aspect of our model may also be an advantage, since not much is known about the basal levels of these active kinases in real biological networks. These pathways are connected, and hence, the active kinase levels can be elevated due to several factors. In order to show a peak response, our model requires elevated initial concentration of active kinase of any one intermediate cycle in the cascade. This supports the fact that any of these cycles may be connected to other networks. Second, the model is not as robust as others that include overt feedback loops, because adaptation is observed only in a narrow range of stimulus parameter at steady state - the OFF-ON-OFF regime. Lastly, the number of cycles needed to show a highly efficient adaptation (maximum amplification) is at least six.

There are several factors that have been shown theoretically to disrupt ultrasensitivity. These include product inhibition, substrate sequestration [41], binding [42], and other approximations in the biochemical kinetics [43]. In principle, we cannot ignore the protein-kinase ($[EY]$) and protein-phosphatase ($[EX]$) concentrations when we consider the effects of sequestration. Blüthgen et al. [41] discuss the effects of sequestration in a typical ultrasensitive cascade and argue that, in the presence of non-negligible $[EY]$ and $[EX]$ concentrations, the phosphatase E' binds rapidly with active form X . This reduces the capability of this active form to stimulate downstream cycles, even if a strong and prolonged stimulus is applied. Since most of the 'target' X is sequestered by the phosphatase, it would desensitize the downstream receptors. This results in a transient time peak response, where signal dies out despite prolonged stimulus (Figure 3A – ref [41]). In our model, we observe the same effect in the OFF-ON-OFF (pre-ultrasensitive) regime, even without the inclusion of sequestration. In the molecular mechanism (Figure 4A–C), we have shown that the presence of a coherent feed-forward loop, which essentially desensitizes the input and forces the amplitude in the time course to reach lower values. In our model, the concentration of the active form X is reduced, not because of the high phosphatase concentration, but because of the insufficient stimulus to sustain the phosphorylated active form. Therefore, the lack of enough stimulus in the OFF-ON-OFF regime produces the same effect as sequestration in terms of eliciting a transient time response. However, a switch-like adaptation response clearly requires that $[EY]$ and $[EX]$ should be negligible. Mathematically, if sequestration were to be included in our model, the transient peak response will be sustained even for high stimulus concentrations, but the three regimes and the steady state limit will disappear.

As they initiated their theoretical work on PD cascades, Goldbeter and Koshland [11] derived an expression for the response coefficient of one PD cycle. Kholodenko et al. [44] then redefined the response coefficient as the fractional change in the activated target brought about by a small fractional change in the input signal. They showed that the overall response coefficient is simply the product of the response coefficients of all the cycles in the cascade. This finding holds true for our model, as well. Alternately, Bruggeman et al. [45] analyzed the global response of several pathways that are linked together, by treating each pathway as a module and then conducting a modular response analysis. It would not be possible to capture the transient time behavior in our model, simply because the modular response analysis captures only the stable steady-state behavior.

Additionally, there are signaling cascades where the phosphorylated protein itself acts as an enzyme for the next cycle [9]. It will be worthwhile to examine such a model for any limiting property induced by the length of the cascade. The behavior of the cascade outside the constraints of ultrasensitivity may also reveal valuable information about *in vivo* pathways. Nevertheless, our findings bring about a new perspective of the complex

properties that a highly simplified, open cascade can elicit. Pathways of this nature that are capable of signal transduction, amplification, and adaptation together—merely through open multistep cascades—could have been the first and foremost designs of the earliest signaling systems prior to the evolution of more robust, closed circuits. We propose that these simple cascades comprising chains of PD cycles, and possibly other types of minimal cascades, could have been the precursors to the finely-tuned and robust contemporary signaling systems. It is likely that evolutionary studies on these types of pathways could yield some extraordinarily valuable information on their origins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Various types of signal transduction cascades

A. A unidirectional MAPK signaling cascade with one single PD cycle and two dual PD cycles. **B.** A schematic representation of a unidirectional open signaling cascade with single PD cycles. A signal is received by a receptor and transmitted to the first PD (phosphorylation–dephosphorylation) cycle, and then propagated downstream by sequentially activating other cycles. **C.** A schematic representation of a closed signaling cascade with single PD cycles. The feedback loop may be either positive or negative.

Figure 2. Steady-state responses of an open signaling cascade as a function of increasing stimuli

A. GK model: X^* is plotted against the stimulus parameter, V_M . **B.** Steady state plot of open cascade model where X_i^* ($i=1,2,3,4,5,6,7$) is plotted against V_M . Note that in the open cascade model, the sharp threshold stimulus is clearly visible at $V_M = 0.832$. **C.** Roots of the steady-state equation of the reduced model. When the response of the downstream cycles coincide, the dynamics can be captured by Equation 9. The steady state of this model has three positive real roots $\beta_1^*, \beta_2^*, \beta_3^*$ in the OFF-ON-OFF regime. **D.** Steady-state plot of the open cascade model and a bistable plot of the reduced model. The thick black line shows the steady state for the primary model (Equation 1). The bistable curve (gray-blue circles) is the steady-state diagram for the reduced model (Equation 9). The filled blue circles indicates the stable steady state and the open green circles indicate the unstable steady state. Pre-ultrasensitive and ultrasensitive regimes are divided by the threshold stimulus V_2^* , whereas V_1^* is the threshold where the OFF-ON-OFF behavior originates. Also note that the OFF states in the pre-ultrasensitive regime and the ON states in the ultrasensitive regime of the primary and reduced models all coincide. Parameters values are as follows: $K=K_i=K_i'=0.008$, $\alpha = 1.5$.

Figure 3. Peak response of a primary open cascade model

A. OFF-ON-OFF regime: ($X_1^0=0.25$) produces a damping effect. **B.** OFF-ON-OFF regime: ($X_1^0=0.35$) produces an amplifying effect. The 7th cycle makes an abrupt jump from a low value to X_{max} , followed by a less abrupt fall back to X_∞^* , thus behaving like a switch (also see inset). The maximum value X_{max} and steady state X_∞^* are close to roots β_3^* and β_1^* respectively of the reduced model (red; see inset) (Equation 9). Note that the time axes are different in A. and B. Parameter values are as follows: $N=7$, $V_M = 0.815$, $\alpha = 1.55$, $K = 0.008$. **C.** ON regime: ($X_{1,...,7}^0=0$) yields a monotone increase to higher steady states. **D.** ON regime: ($X_1^0=0.3$, $X_{2,...,7}^0=0$) produces a transient peak before settling at higher steady states.

Figure 4. Molecular mechanisms giving rise to temporal switch-like and adaptation behaviors

A. In the OFF state, there is a basal rate of $Y P_1$ production, yet there remains an insufficient yield to activate the feed-forward module (FFM). **B.** Schematic of relative concentrations of species present in the OFF state. **C.** In the OFF-ON transition, a feed-forward module is active which is stimulated by the presence of a basal (or initial) fraction of the active form ($Y P_1$) generated by cycle 1. Due to excessive $Y P_2$, the phosphatase E_2 is saturated and dephosphorylation is inhibited. Together with the active FFM and saturation inhibition of E_2 , switching of OFF-ON occurs abruptly. **D.** Schematic of relative concentrations of species present during the switching from the OFF to ON state. **E.** After the ON state is reached, it is maintained for a prolonged period of time due to the balance between the rates

of the FFM and dephosphorylation reaction. **F.** Schematic of relative concentrations of species present during maintenance of the ON state, with a diminished level of YP_1 . **G.** Once the level of YP_1 is depleted, the FFM is broken, leaving the phosphatase activity alone to switch off the cascade, less abruptly than it was activated. **H.** Schematic of relative concentrations of species present in the OFF state.

Figure 5. Relative abundance of initial active protein and cascade-length requirements for the OFF-ON-OFF regime

A. Maximum activity during the time course X_i^m with respect to the fraction of initial active protein elicited from PD cycle 1. X_i^m shows a graded response for $N=2$, and an increasingly switch-like response for $N=7$. Parameters $V_M = 0.82$, $K = 0.008$, $\alpha = 1.55$. **B.** As V_M increases, the fraction of initial active protein necessary to push the system to the OFF-ON-OFF behavior decreases. **C.** Shows the corresponding downstream cycle—or cascade length—required for OFF-ON-OFF behavior to originate as a function of stimulus (V_M) strength.

Figure 6. Dependence of the three different behavioral regimes on model parameters
A. As the coupling strength α increases, the OFF-ON-OFF regime widens ($K=0.01$). **B.** As K decreases, the OFF-ON-OFF regime widens ($\alpha=1$), and as K increases, ultrasensitive behavior is disrupted.

Table 1

Parameters for the model Equation 1

Parameter ($i=1,2,\dots,N$)	Description	Values
V_{Mi}	Maximum rate of the phosphorylation reaction	as described
V'_{Mi}	Maximum rate of the dephosphorylation reaction	1
K_i	Michaelis–Menten constant for phosphorylation process	0.01
K'_i	Michaelis–Menten constant for dephosphorylation process	0.01
α_i	Strength of feed-forward activation	1