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Modeling Species-Specific Diacylglycerol Dynamics in the RAW 264.7 Macrophage

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Abstract

A mathematical model of the G protein signaling pathway in RAW 264.7 macrophages downstream of P2Y₆ receptors activated by the ubiquitous signaling nucleotide uridine 5'-diphosphate is developed. The model, which is based on time-course measurements of inositol trisphosphate, cytosolic calcium, and diacylglycerol, focuses particularly on differential dynamics of multiple chemical species of diacylglycerol. When using the canonical pathway representation, the model predicted that key interactions were missing from the current network structure. Indeed, the model suggested that accurate depiction of experimental observations required an additional branch to the signaling pathway. An intracellular pool of diacylglycerol is immediately phosphorylated upon stimulation of an extracellular receptor for uridine 5'-diphosphate and subsequently used to aid replenishment of phosphatidylinositol. As a result of sensitivity analysis of the model parameters, key predictions can be made regarding which of these parameters are the most sensitive to perturbations and are therefore most responsible for output uncertainty.

Keywords

Cellular Signaling Pathways; Mathematical Modeling; Sensitivity Analysis; P2Y₆ Receptor; Uridine 5'-Diphosphate

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Introduction

Strong selection advantages are provided to any organism with the ability to send, receive and interpret information from the surrounding environment. In addition, evolution from unicellular to multi-cellular organisms required the development of an elaborate communication network between individual cells. These networks typically rely on the release and subsequent binding of signaling molecules. The effects of the signals on the target cells are very broad; responses can include survival, chemotaxis, proliferation, and programmed cell death (apoptosis). The processes of ligand detection through binding to specific receptor proteins and subsequent evolution of responses are critical aspects of cellular signaling which determine cellular development and behavior. The primary focus of this work is to investigate the differential mechanisms of production and degradation of specific chemical species of diacylglycerol (DAG) as a means of diagnosing a particular signaling pathway model. It also provides an expanded model of a G protein coupled receptor pathway that incorporates a quantitative analysis of an important category of lipid molecular species that function as cellular second messengers.

Although nucleotides serve as some of the most basic building blocks of ribonucleic acids (RNA) and deoxyribonucleic acid (DNA), they can also act as important signaling molecules through their action on the family of P2 nucleotide receptors (Korcok et al., 2005). Nucleotides such as adenosine triphosphate (ATP) and uridine triphosphate (UTP) are known to regulate an array of diverse biological effects upon their release from cells during inflammation, mechanical stimulation or disruption (Born and Kratzer, 1984; Grygorczyk and Hanrahan, 1997; Lazarowski and Harden, 1999; You et al., 2002). Once released into the extracellular fluid, ATP and UTP are degraded by ecto-nucleotidases into adenosine diphosphate (ADP) and uridine diphosphate (UDP), respectively (Connolly and Duley, 1999; Hou et al., 2002; Zimmermann et al., 1998). UDP then serves as the primary agonist for the P2Y₆ G protein-coupled receptor (GPCR) (Nicholas et al., 1996), which has been shown to couple to the G α_q isoform (Yoshioka et al., 2001) of the associated G protein.

This work focuses on modeling the signaling events downstream of UDP activation of the P2Y₆ receptor in RAW 264.7 macrophages. There have been a number of models developed to study GPCR signaling pathways in a variety of cell types (Garrad et al., 1998; Lemon et al., 2003; Mishra and Bhalla, 2002; Soto and Othmer, 2006; Woolf and Linderman, 2003), however, as noted by the Alliance for Cellular Signaling (2008), the RAW 264.7 macrophage has many properties that are desirable, including the fact that they express receptors for numerous different ligands, which led to the decision to focus on this cell line. Although a number of mathematical models have helped to uncover complex interactions in the areas of the G protein cascade (Heinrich et al., 2002; Mahama and Linderman, 1994; Tang and Othmer, 1995; Yi et al., 2003) and calcium (Ca²⁺) flux (De Young and Keizer, 1992; Flaherty, 2007; Keizer and De Young, 1992; Li and Rinzel, 1994; Wagner and Keizer, 1994), there are currently no models that address changes in DAG at the chemical species level after stimulation of GPCRs with ligands.

DAGs serve as second messengers through the activation of protein kinase C, an enzyme linked to the regulation of many cellular processes including cell differentiation, proliferation, carcinogenesis, development, and memory in multiple mammalian cell types (Bishop and Bell, 1988; Dekker and Parker, 1994; Mochly-Rosen, 1995; Newton, 1995; Nishizuka, 1995). Increases in intracellular concentrations of DAG are also believed to contribute to the transduction of mitogenic signals (Habenicht et al., 1981; Magnaldo et al., 1986; Pessin et al., 1990; Raben et al., 1987; Sasaki and Hasegawa-Sasaki, 1985) as well as secondary secretion and aggregation (Werner et al., 1991). More than 50 different chemical species of DAG have been identified, which differ in acyl chain length and degree of unsaturation. With evidence

for differential roles of these species in cellular processes (Deacon et al., 2002; Pettitt et al., 1997), determining the species-specific regulation of DAG in the signaling process is crucial to obtaining a comprehensive understanding of how the cell responds to its stimulus. Our model therefore places a major emphasis on the study of species-specific DAG dynamics. While our model is capable of simulating all 28 different species of DAG for which experimental data has been obtained (with each species indexed by a superscript i , $i = 1 \dots 28$, in the Model Structure section), to illustrate how the concentrations change within the cell, results are shown only for two DAG species ($i = 1, 2$). Although the mechanisms of species-specific DAG production and degradation downstream of agonist stimulation of P2Y receptors are still relatively unclear, our modeling efforts are leading to novel pathway propositions.

Sensitivity analysis of model parameters provides further insight into the parameters most responsible for the uncertainty in model output. Such analysis can therefore lead to key insights into the model structure and also pinpoint areas of future experimental focus.

Model Structure

Deriving a Mathematical Model of the Key P2Y₆ Pathway Components

The model consists of a system of nonlinear ordinary differential equations and is separated into four modules: Receptor Dynamics (two equations), G protein Cascade (three equations), DAG Production and Degradation (initially a single separate equation, indexed by a superscript i , for each DAG species modeled; later represented by two equations for each species to reflect the assumption that there are two separate pools of DAG production within the cell), and Cytosolic Calcium Dynamics (three equations). Where appropriate, the number of molecules of species X will be denoted by N_X , whereas $[X]$ will denote the concentration of X in μM .

Module 1: Receptor Dynamics—The representation of P2Y₆ receptor dynamics is based on previous work by Lemon et al. (2003), which provides a detailed account of receptor regulation for the P2Y₂ receptor in response to its primary agonist UTP (Nicholas et al., 1996). In the case of the P2Y₆ receptor, the mechanisms are assumed to be similar to those of P2Y₂, only Brinson and Harden (2001) observed a much slower rate of desensitization for the P2Y₆ receptor in response to its primary agonist UDP. To account for this slower rate of desensitization, the receptor phosphorylation rate, k_p , is modified to fit the P2Y₆ observations of Brinson and Harden.

GPCR activity is regulated by several processes, including phosphorylation and subsequent uncoupling from G proteins or internalization, which causes desensitization. The phosphorylated receptors can then be dephosphorylated, returning to the surface of the plasma membrane. Our model assumes that initial concentration of ligand is sufficiently high so that it is not significantly depleted by binding to the receptors and therefore can be presumed as a fixed concentration. Assuming the kinetics of ligand binding are fast relative to the other processes in the model (Wagner and Keizer, 1994), Lemon et al. arrive at the following reduced two-variable system, leaving only the slow kinetics of receptor regulation (2003):

$$\frac{dN_{R^S}(t)}{dt} = k_r N_{R_T} - \left(k_r + \frac{k_p [L]}{K_1 + [L]} \right) N_{R^S} - k_r N_{R_P^S} \quad (1)$$

$$\frac{dN_{R_P^S}(t)}{dt} = [L] \left(\frac{k_p N_{R^S}}{K_1 + [L]} - \frac{k_e N_{R_P^S}}{K_2 + [L]} \right), \quad (2)$$

where $[L]$ denotes the ligand concentration and N_{R^S} ($N_{R_p^S}$) represents the number of ligand-bound and unbound unphosphorylated (phosphorylated), and therefore “active” (“inactive”), surface receptors. Lemon also takes into account the number of internalized receptors, N_I , so that $N_{R_T} = N_{R^S} + N_{R_p^S} + N_I$ is the fixed total number of receptors in the cell. The unphosphorylated and phosphorylated receptor dissociation constants are given by K_1 and K_2 , respectively. Here ligand bound surface receptors are phosphorylated irreversibly at a rate k_p . Upon phosphorylation, receptors are internalized at a rate k_e . Internalized receptors can then undergo dephosphorylation and return to the surface at a rate k_r .

Module 2: G protein Cascade—Once a P2Y₆ receptor becomes activated via ligand binding, it undergoes a conformational change (a change in shape), allowing a G protein to bind to the activated receptor. Heterotrimeric G proteins are receptor activated proteins that are bound to the inside surface of the cell membrane. They consist of three subunits: $G\alpha$, and tightly associated $G\beta$ and $G\gamma$. Once bound to an activated receptor, the G protein releases its bound nucleotide, guanosine diphosphate (GDP), from the $G\alpha$ subunit, allowing association with a new molecule of guanosine triphosphate (GTP). This nucleotide exchange promotes the dissociation of the GTP-bound $G\alpha$ subunit, the $G\beta\gamma$ dimer, and the GPCR. Although both $G\alpha$ -GTP and $G\beta\gamma$ can then activate different signaling pathways and effector proteins, we focus only on the reactions downstream of $G\alpha$ -GTP activation. Eventually the $G\alpha$ subunit will hydrolyze the GTP to GDP through its inherent enzymatic activity. This allows $G\alpha$ to reassociate with $G\beta\gamma$, starting a new cycle. A simplified model of the time-dependent production and degradation of $G\alpha$ -GTP, denoted by G^* , is

$$\frac{dN_{G^*}(t)}{dt} = k_a N_{R^S} (N_{G_T} - N_{G^*}) - k_d N_{G^*}, \quad (3)$$

where, according to mass action kinetics, we have assumed that G^* are produced at a rate proportional to the number of activated surface receptors, N_{R^S} , and the number of inactive G proteins, $N_{G_T} - N_{G^*}$, where N_{G_T} is the total number of G proteins, both activated and inactivated. The second term in Eq. (3) represents the deactivation rate of G proteins by hydrolysis of GTP into GDP and is proportional to the current number of active G proteins. In this case, as laterally diffusing $G\alpha$ -GTP subunits bind isoforms of inactive (presumably freely diffusing) cytosolic enzymes known as phospholipase C (PLC) on the inner leaflet of the plasma membrane, $G\alpha$ -GTP-PLC complexes are formed.

The bound state complex $G\alpha$ -GTP-PLC is considered fully activated when bound to calcium (Ca^{2+}), where it then hydrolyzes plasma membrane-bound phosphatidylinositol bisphosphate (PIP_2) molecules into inositol trisphosphate (IP_3) and DAG. For simplicity, we assume that the number of $G\alpha$ -GTP-PLC complexes is directly proportional to the number of G^* , with proportionality constant λ , and PIP_2 replenishment occurs through a phenomenological term linear in the difference from the initial amount, $N_{PIP_2}(0)$. Therefore we can write the time dependence of PIP_2 as

$$\begin{aligned} \frac{dN_{PIP_2}(t)}{dt} = & -k_{hyd} \left(\frac{[Ca^{2+}]}{K_C + [Ca^{2+}]} \right) \lambda N_{G^*} N_{PIP_2} \\ & + k_{rep} (N_{PIP_2}(0) - N_{PIP_2}), \end{aligned} \quad (4)$$

where the effective hydrolysis rate is proportional to the number of PIP_2 and G^* molecules, and we assume rapid binding kinetics of cytosolic Ca^{2+} (in μM) to the activated $G\alpha$ -GTP-PLC

complex, which leads to the Michaelis-Menton type term in the equation. Here K_C represents the dissociation constant for the Ca^{2+} binding site on the PLC molecule.

The generation of IP_3 is determined by the hydrolysis rate of PIP_2 . As IP_3 molecules are known to diffuse through the cytosol, we refer to IP_3 production and degradation in terms of concentration (in μM) instead of number of molecules. We assume that IP_3 is degraded at a rate proportional to the current concentration of IP_3 . The equation for IP_3 is therefore

$$\frac{d[\text{IP}_3](t)}{dt} = k_{\text{hyd}} \left(\frac{[\text{Ca}^{2+}]}{K_C + [\text{Ca}^{2+}]} \right) \lambda N_{G^*} \left(\frac{10^6}{N_{\text{av}} \cdot v} \right) N_{\text{PIP}_2} - k_{d3} [\text{IP}_3], \quad (5)$$

where v is the volume of the cell (in L) and N_{av} is Avogadro's constant.

Module 3: Diacylglycerol production and degradation—The main focus of this work is on modeling the production and degradation of different species of DAG. As stated above, it is well-known that early DAG production is a result of PIP_2 hydrolysis at the plasma membrane (Jolly et al., 1996; Wright and McMaster, 2002; Wright et al., 1988), where it is produced stoichiometrically with IP_3 . Each DAG species, indexed by a superscript i ($i = 1 \dots 28$), can then be converted into other molecules via several pathways. For example, a diacylglycerol kinase can phosphorylate DAG into phosphatidic acid (PA). The effects of these pathways are combined into one effective rate constant for each DAG species, k_{dp1}^i , so that the rate at which the i^{th} DAG species is lost is proportional to its current concentration. Using this canonical pathway to describe total species-specific DAG production and degradation in the cell results in an equation very similar to that of IP_3 :

$$\frac{d[\text{DAG}^i](t)}{dt} = k_{\text{hyd}} \left(\frac{[\text{Ca}^{2+}]}{K_C + [\text{Ca}^{2+}]} \right) \lambda N_{G^*} \left(\frac{10^6}{N_{\text{av}} \cdot v} \right) N_{\text{PIP}_2} - k_{dp1}^i [\text{DAG}^i]. \quad (6)$$

DAG quantification was performed according to Callender et al. (2007), which allows for the simultaneous quantification of up to 28 different chemical species of DAG, that are experimentally measured and for which adequately similar chemical standards exist. We found that the responses of many DAG species cannot be modeled via this signaling pathway alone (see Fig. 1A and 1B). In fact, many mono- and di-unsaturated DAGs such as 34:1 DAG (containing 34 carbons and 1 double bond), shown in Fig. 1B, exhibit a biphasic response in cells with 25 μM UDP stimulation.

These data are consistent with the existence of two separate pools of DAG for each i^{th} DAG species, denoted by the subscripts $p1$ for pool 1 and $p2$ for pool 2: DAG_{p1}^i , originating from PIP_2 hydrolysis post-agonist stimulation and degraded as described above, and an intracellular pool, DAG_{p2}^i , present in high concentrations in resting cells, that is rapidly converted to PA upon UDP stimulation via a DAG kinase. The latter reaction creates an initial decrease in DAG_{p2}^i levels. Each DAG species has varying levels of representation in these two pools. For instance, the second pool contains more of the mono- and di-unsaturated DAG species, whereas the species produced from early hydrolysis of PIP_2 are enriched in polyunsaturated fatty acid-containing (PUFA-containing) species.

All measured DAG species exhibit a second rise phase, peaking on average around 15 minutes. Although this later rise phase has in some cases been attributed to phospholipase D (PLD) activity (Jolly et al., 1996; Wright and McMaster, 2002; Wright et al., 1988), RAW 264.7 cells

do not demonstrate PLD activation upon stimulation by UDP (data not shown). Thus a hypothetical mechanism for the observed response is, in addition to early production from PIP₂ hydrolysis, the activation of a known DAG metabolic pathway: agonist stimulated conversion of an intracellular pool of DAG to PA followed by conversion to cytidine diphosphate-DAG (CDP-DAG) and subsequently to phosphatidylinositol (PI). This results in a transformed pool of PIP₂ from the original primarily polyunsaturated form. A schematic of this two-pool DAG model is shown in Fig. 2. In this scenario each DAG_{p2}ⁱ is replenished by a phosphatidylcholine-specific enzyme while it is simultaneously used for PI production and replenishment of the PIP₂ pool. The revised equation for DAGⁱ produced from PIP₂ hydrolysis based on this two-pool model is similar to Eq. (6) but includes an additional term to account for a contribution from the second pool of DAGⁱ. The proposed model can simultaneously account for the two pools of each of the 28 measured DAG species. Individual DAG species, indexed by *i* (*i* = 1 ... 28), are each modeled with equations of the same form (Eqs. (7) and (8) below, for pool 1 and pool 2, respectively), but may have different rates of production and degradation. Therefore, the relevant rate constants in Eqs. (7) and (8) also contain the same superscript, *i*. This complexity is reflected by the fact that although pool 1 DAG is predominantly polyunsaturated and pool 2 DAG is monounsaturated, the rate kinetics within each pool may not be monolithic. The equation for DAG_{p1}ⁱ of the *i*th DAG species, denoted DAG_{p1}ⁱ, is therefore given by

$$\begin{aligned} \frac{d[\text{DAG}_{p1}^i](t)}{dt} &= k_{hyd} \left(\frac{[\text{Ca}^{2+}]}{K_C + [\text{Ca}^{2+}]} \right) \lambda N_{G^*} \left(\frac{10^6}{N_{av} v} \right) N_{PIP_2} \\ &\quad - k_{dp1}^i [\text{DAG}_{p1}^i] + \eta^i [L] k_{dp2}^i [\text{DAG}_{p2}^i], \end{aligned} \quad (7)$$

where η^i denotes the fraction of DAG_{p2}ⁱ contributing to DAG_{p1}ⁱ, and k_{dp2}^i is the degradation rate of DAG_{p2}ⁱ. To account for the dynamics of the second pool of DAG in the *i*th DAG species, denoted DAG_{p2}ⁱ, we also include the equation

$$\frac{d[\text{DAG}_{p2}^i](t)}{dt} = -[L] k_{dp2}^i [\text{DAG}_{p2}^i] + k_{ap2}^i ([\text{DAG}_{p2}^i](0) - [\text{DAG}_{p2}^i]), \quad (8)$$

where ligand-dependent phosphorylation of DAGs from pool 2 occurs at a rate proportional to the current concentration of DAG_{p2}ⁱ for the *i*th DAG species. Pool 2 DAGs are then replenished at a rate proportional to the amount of DAG_{p2}ⁱ that has been phosphorylated by DAG kinase.

Module 4: Cytosolic Calcium dynamics—Upon receptor activation, PIP₂-derived IP₃ diffuses through the cytosol and binds to IP₃-gated Ca²⁺ channels (IP₃Rs) on the endoplasmic reticulum (ER), releasing Ca²⁺ into the cytosol. Li and Rinzel (1994) developed a simplified model of cytosolic Ca²⁺ dynamics which allows for Ca²⁺ exchange between the cell and the extracellular medium. Due to the minimalistic yet thorough approach of Li and Rinzel and the ability of their model to explain various experimental data, we have chosen to utilize this model of Ca²⁺ dynamics in our system. Thus cytosolic Ca²⁺ dynamics are modeled by the following equations:

$$\frac{d[Ca^{2+}](t)}{dt} = [\nu_1 m_{\infty}^3 (h)^3 + \nu_2] \times [[c_0] - (1+c_1)[Ca^{2+}]] - \frac{\nu_3[Ca^{2+}]^2}{k_{er}^2 + [Ca^{2+}]^2} + \varepsilon \left[j_{in} - \frac{\nu_4[Ca^{2+}]^2}{k_{pl}^2 + [Ca^{2+}]^2} \right] \quad (9)$$

$$m_{\infty} = \left(\frac{[IP_3]}{[IP_3] + d_{IP_3}} \right) \left(\frac{[Ca^{2+}]}{d_{act} + [Ca^{2+}]} \right) \frac{dh(t)}{dt} = a ([Ca^{2+}] + d_{inh}) \left[\left(\frac{d_{inh}}{[Ca^{2+}] + d_{inh}} \right) - h \right] \quad (10)$$

$$\frac{d[c_0]}{dt} = \varepsilon \left[j_{in} - \frac{\nu_4[Ca^{2+}]^2}{k_{pl}^2 + [Ca^{2+}]^2} \right]. \quad (11)$$

Here $h(t)$ represents the fraction of IP_3 receptors not yet inactivated by Ca^{2+} binding to the inactivation site of the IP_3R , whereas m_{∞} represents the probability of the IP_3R being in the activated (open) state, with IP_3 bound as well as Ca^{2+} bound to the activation site. Although the IP_3R contains four subunits, De Young and Keizer found that a power close to three for each of these terms provided the best fit to experimental data (1992). The c_0 term represents total free Ca^{2+} per cytosolic volume, and the parameter c_1 is the volume ratio between the ER and the cytosol.

The rate parameters ν_1 , ν_2 , and ν_4 are effective constants for the permeability of IP_3 channels, ER membrane leakage, and Ca^{2+} pump permeability, respectively. The dissociation constants for the Ca^{2+} activation and deactivation sites on the IP_3R are d_{act} and d_{inh} , respectively, and a is the IP_3R binding constant for Ca^{2+} -dependent inhibition. The parameter k_{pl} is the Michaelis constant for Ca^{2+} flux out of the cell across the plasma membrane, while ε is the ratio of the total area of the plasma membrane to the total area of the ER.

All Ca^{2+} module parameters listed above were taken from either Li and Rinzel (1994) or De Young and Keizer (1992), while the following parameters were estimated to obtain a best fit to the experimental data in Fig. 1D: The dissociation constant for the Ca^{2+} binding site on the PLC molecule, K_C , adjusts the Ca^{2+} peak time, while the maximum ER Ca^{2+} pump rate, ν_3 , the Michaelis constant for Ca^{2+} uptake from the cytosol into the ER, k_{er} , and the receptor dissociation constant for the IP_3 receptor, d_{IP_3} , all control the Ca^{2+} peak height and shape. The parameter representing a constant flux of Ca^{2+} through the plasma membrane and into the cell, j_{in} , sets the level of Ca^{2+} at later time points, and its value was chosen to lie in the range of values chosen by Li and Rinzel (1994).

For a more detailed account and analysis of the role of Ca^{2+} in this pathway in RAW 264.7 cells, see Flaherty (2007).

Methods

Time-Course Experiments

Diacylglycerol lipid analysis - Stimulation of RAW 264.7 cells with 25 μ M UDP, phospholipid extraction, and diacylglycerol separation and quantification were performed according to the procedures described in Callender et al. (2007).

IP₃ Binding assay - Lysates from RAW 264.7 cells were assayed using the [³H] Biotrak assay kit from GE Healthcare (Buckinghamshire, UK), based on a competitive radioreceptor assay (Palmer et al., 1989). RAW 264.7 cells were grown in 60 mm dishes and stimulated as described in Callender et al. (2007). Cells were washed with 2 ml ice-cold PBS, aspirated and lysed by scraping in 250 µl 5.4% perchloric acid on ice. Cell lysates were transferred to 1.5 ml microfuge tubes and assayed according to the manufacturer's instructions. [IP₃] is calculated as pmols/100 µl lysate.

Intracellular free calcium assay - Data were obtained by measuring the fluorescent emission at 538 nm of RAW 264.7 cells loaded with fluo-3 acetoxymethyl (Molecular Probes, Carlsbad, CA). Fluorescence measurements were performed using a Fluoroskan Ascent microplate fluorometer with the Ascent software (Thermo-Labsystems, Waltham, MA), according to AfCS Procedure Protocol PP00000176, on the AfCS website (2008). Intracellular free calcium data for RAW 264.7 cells stimulated with 25 µM UDP were obtained from the AfCS single ligand screen (Natarajan et al., 2006), and are also available on the AfCS website.

Numerical Analysis

Our system of ordinary differential equations was integrated using the MATLAB solver ode15s, which is a variable order solver based on the numerical differentiation formulas (NDFs) (Shampine and Reichelt, 1997). The system used includes multiple species of DAG and reflects that, in the two-pool model, all species are found in both pools. Once an exhaustive search through the literature for known rate parameters was performed, unknown rates were estimated using SIMULINK, which estimates the parameters by minimizing a user-specified cost function via a user-specified optimization method (The Mathworks, 2008). We chose the nonlinear least squares optimization method of Levenberg-Marquardt to minimize a sum of squared errors cost function of the empirical observations and model predictions for IP₃, Ca²⁺, and multiple species of DAG.

Sensitivity Analysis

A sample matrix of the space of γ input variables (i.e., γ = number of model parameters), consisting of ten percent variations from each parameter's nominal value, was generated using the Latin Hypercube Sampling (LHS) method, with a sample size of N (here, we chose $N = 5000$). The LHS sample matrix was found using the coding environment SIMLAB 3.0.x. The sample, $M_{\alpha\beta}$, $\alpha = 1, \dots, N$ and $\beta = 1, \dots, \gamma$, was then propagated through the mathematical model to form a vector of outputs, y_{α} , $\alpha = 1, \dots, N$. To obtain a quantitative global sensitivity analysis, the measure of standardized regression coefficients (SRCs) determined from fitting the response surface was utilized. The slopes, R_y^2 , from the multiple regression model and the corresponding SRCs were then computed for each variable in the model and for each time point using the MATLAB *regress* function. R_y^2 values were used to determine the degree of nonlinearity in the model output with respect to the rate parameters and therefore the extent to which the SRCs could provide accurate measures of sensitivity. In general, SRCs are considered to be appropriate sensitivity indicators when R_y^2 values are ≥ 0.7 (Saltelli et al., 2004).

Results

Estimation of Unknown Rate Parameters from Experimental Results

With the exception of the calcium module variables, initial conditions for each model variable are chosen to reflect the levels of each variable over baseline levels for a step application of ligand such that $[L](t) = 0$ for $t < 0$ and $[L](t) = [L]$ for $t \geq 0$. Thus, we set $N_{RS}(0) = N_{RT}$. Also, since PIP₂ and DAG_{p2} are initially at their maximum value, we have $N_{PIP_2}(0) = N_{PIP_2T}$ and

$N_{DAG_{p2}}(0) = N_{DAG_{p2T}}$. Phosphorylated surface receptors, R_p^S , activated G proteins, IP_3 , and DAG_{p1} are all given an initial value of zero since we assume there is no production of these molecules before receptor activation. Although the initial concentration of calcium, $0.016 \mu M$, is taken from experimental data, the initial values of the remaining two calcium module variables, $h = 0.8$ and $c_0 = 2 \mu M$, are chosen to reflect values given by Li and Rinzel (1994).

Experimental observations of $P2Y_6$ receptor dynamics from Brinson and Harden in 1321N1 astrocytoma cells (2001), IP_3 and Ca^{2+} measurements, and species-specific DAG measurements of the response to $25 \mu M$ UDP in RAW 264.7 cells are used to compare with the simulations obtained from the system of ten nonlinear ordinary differential equations described above (Eqs. (1)–(5) and Eqs. (7)–(11)). These data are also used for the estimation of unknown rate parameters. Table 1 includes a list of all model parameters, their values, and a reference for those taken from the literature. Of these 34 parameters, 18 are taken from the literature, 2 are directly measured, and 14 are estimated from experimental data.

Although the mathematical model can describe the temporal evolution of DAG production and degradation of all 28 different species of DAG experimentally measured, to simplify the illustration, we display simulation results displaying differential characteristics representative of two types of DAG species, PUFA-containing DAGs and mono- and di-unsaturated fatty acid-containing DAGs. Time-series data for the 38:4 DAG and 34:1 DAG responses to $25 \mu M$ UDP are shown in Figs. 1A and 1B, respectively. Solid gray lines are derived from numerical simulations of our initial DAG model (simultaneously using separate equations in the form of Eq. (6) for each DAG species, with different values of k_{dp1}^i for each species' equation), whereas solid black lines are derived from numerical simulations of the combined effect of DAG_{p1}^i and DAG_{p2}^i from pools 1 and 2, as described above. As the method used for quantification of DAG species measures change in total cellular DAG species levels over baseline levels, DAG^i response is simulated by adding the outputs from Eq. (7) and Eq. (8) for the i^{th} DAG species. Data points and error bars correspond to experimental values obtained using the DAG assay as described in the methods section.

Although the second and larger rise phase is observed in most of the DAG species, the early kinetic behavior, within the first three minutes of stimulation, varies quite drastically between PUFA-containing DAGs and mono- and di-unsaturated DAGs. PUFA-containing DAGs, such as 38:4 DAG, are consistently seen as increasing immediately upon stimulation, whereas mono- and di-unsaturated species, such as 34:1 DAG, display an immediate and small peak (~ 43 ng for 34:1 DAG) two minutes after stimulation and then exhibit a brief yet substantial recovery by 3 minutes which is followed by a larger production (~ 104 ng for 34:1 DAG) at 15 minutes. By the 30 minute time point, all DAG species have begun the process of slow recovery toward baseline levels.

In approximately 30% of the experimental cases, total DAG levels in many of the mono- and di-unsaturated species are seen to actually decrease significantly below baseline levels within the first 3 minutes of stimulation (data not shown). Although the reason for this variation in response could not be determined, according to the two-pool model structure, this would suggest a more rapid phosphorylation of mono- and di-unsaturated DAG_{p2} such that initial production of mono- and di-unsaturated DAG_{p1} is more than offset by early conversion of this type of DAG_{p2} resulting in a net loss of total mono- and di-unsaturated DAG until enough mono- and di-unsaturated DAG_{p2} has been converted to aid in PIP_2 replenishment. By increasing the value of mono- and di-unsaturated k_{dp2} , our model can accurately reproduce this kinetic behavior.

As seen in Fig. 1, different species of DAG respond differently to P2Y₆ stimulation, suggesting different rates of conversion or degradation from both pools as well as different initial values and rates of production of DAG from the second pool. The rate of degradation of

DAG_{p2}ⁱ, k_{dp2}^i (where, as mentioned earlier, the superscript *i* refers to a particular species of DAG), appears to control the early behavior of different DAG species. Specifically, for species showing an early rise phase followed by a recovery to baseline by ~ 3 minutes, such as 34:1

DAG (Fig. 1B), k_{dp2}^i sets the level and time of recovery. The degradation rate of

DAG_{p1}ⁱ, k_{dp1}^i , and production rate of DAG_{p2}ⁱ, k_{ap2}^i , set the size and time of the peak observed at later time points. As η^i represents the fraction of DAG_{p2}ⁱ contributing to the resynthesis of

DAG_{p1}ⁱ as described in the two-pool model (Fig. 2), this parameter controls the overall magnitude of DAG response throughout the timecourse. The initial concentration of resting

DAG_{p2}ⁱ levels in the RAW 264.7 cell, $N_{DAG_{p2}^i}$, was estimated from the methods developed in Callender et al. (2007).

Fig. 1C displays the total increase of IP₃ over baseline levels in response to 25μM UDP over a timecourse of 30 minutes. The solid line represents the solution to Eq. (5) with initial condition $N_{IP_3}(t) = 0$, as we assume no change in number of IP₃ molecules for times $t \leq 0$ (i.e., before ligand addition). Data points with error bars are experimental values obtained from the IP₃ assay described in the methods section. Results show that IP₃ peaks within the first 3–4 minutes (~9 pmols) and remains above baseline levels throughout the tested timecourse, suggesting prolonged stimulation of receptors and hydrolysis of PIP₂ molecules.

PIP and PIP₂ species were measured by mass spectrometry (according to Milne et al., 2005) at the same time points and UDP stimulation conditions as DAG species (data not shown). Three independent experiments were performed each in triplicate. Normalization was achieved in respect to two internal standards (37:4 PIP and 37:4 PIP₂). Five PIP species (34:1, 36:2, 38:5, 38:4 and 38:3) and four PIP₂ species (36:2, 38:5, 38:4 and 38:3) were measured consistently with signal-to-noise (S/N) ratios over 3. None of these species were found to be changing with UDP stimulation.

The number of PIP₂ molecules in the cell was estimated by mass spectrometry analysis using 16:0 PIP₂ as an internal standard. The rate of IP₃ degradation, k_{d3} , controls the IP₃ peak time and height observed from the experimental data, whereas the rates of receptor phosphorylation, k_p , effective PIP₂ hydrolysis, $k_{hyd} (= k_{hyd}\lambda)$, and PIP₂ replenishment, k_{rep} , were all used to fit the experimental observations of IP₃ and multiple species of DAG. The parameter k_p sets the height of recovery at later time points in both IP₃ and DAG. The overall magnitude of DAG during the timecourse and the shape of the IP₃ response are controlled by k_{hyd} , whereas k_{rep} sets the size of the DAG response after the initial peak and the peak height of IP₃.

Predictions for the remaining model variables, including activated and inactivated P2Y₆, activated G proteins, and available PIP₂, are obtained by solving Eq. (1) through Eq. (4). Fig. 3 shows model simulations for these four remaining variables, all in response to 25 μM UDP.

Sensitivity Analysis

Model parameter sensitivities are computed as described in the Materials and Methods section, using Standardized Regression Coefficients (SRCs) obtained from fitting a response surface. For all model outputs of interest and all time points from 0 to 30 minutes, the multiple regression model slopes, R_y^2 , are greater than 0.8 and in most cases are very close to one, ensuring that the SRCs are good measures of sensitivity (Saltelli et al., 2005, 2004). As different input variables (rate parameters) affect the model output differently at different times, certain parameters are

seen to be more sensitive at early time points, whereas others are more sensitive at later time points. The time-dependent SRCs corresponding to the most sensitive parameters and the R_y^2 values for IP_3 and $DAG = DAG_{p1} + DAG_{p2}$ are shown in Fig. 4 and Fig. 5, respectively.

Fig. 4 reveals that early IP_3 output is most sensitive to changes in the rate of degradation of IP_3 , k_{d3} , which has a negative effect, and the PIP_2 replenishment rate, k_{rep} , which has a positive effect. At early time points (0 to 550 seconds) the receptor recycling rate, k_r , shows a slight negative effect; however, from 900 to 1800 seconds, k_r becomes the parameter with the strongest positive influence on IP_3 . The parameter most negatively affecting IP_3 production at later time points is the rate of receptor phosphorylation, k_p .

As shown in Fig. 5, the dynamics of each DAG species are mostly driven by the DAG kinetic rates of activation and degradation. Output of DAG is most sensitive to the rate of degradation of DAG_{p2} , k_{dp2} , for the first ~ 400 seconds after stimulation, with this parameter having a negative influence on DAG production. Although the receptor phosphorylation rate, k_p , shows a small yet significant negative effect at later time points, the most sensitive parameter with negative effects from 400 to 1800 seconds is the DAG_{p1} degradation rate, k_{dp1} . The parameters with the strongest positive influence are the DAG_{p2} production rate, k_{ap2} , and the receptor recycling rate, k_r .

From the sensitivity analysis k_{dp2} , k_{ap2} , and k_{dp1} were determined to be the most sensitive parameters for DAG output from 0 to 1800 seconds. Tests were conducted by varying each of these parameters by 50% about their nominal values given in Table 1. (Figs. 6A, 6B, and 6C, respectively.) The rate of phosphorylation in the second pool of DAG, k_{dp2} , greatly affects the level of early decrease in DAG levels over baseline. It is the rates of pool 2 DAG replenishment and pool 1 DAG degradation which greatly impact the late DAG behavior, and the effects of variations in these two rates are in opposite directions of one another, as reflected by the SRC graph from Fig. 5. Indeed, a *rise* in the activation rate k_{ap2} causes an overall increase in DAG and a slower recovery to baseline at later time points, where a similar DAG increase is found by a *decrease* in the degradation parameter k_{dp1} .

Sensitivity analysis on the Ca^{2+} module parameters is not provided as this is beyond the scope of this paper.

Use of Inhibitors to Test the Model

Pharmacological inhibitors of specific enzymes in the proposed pathway were applied as a form of model validation, and the data are in strong agreement with model predictions. Tested inhibitors of DAG metabolism include: U73122, an inhibitor of PI-PLC activity; U73433, the inactive analog of U73122; propranolol, an inhibitor of the conversion of PA to DAG via lipid phosphate phosphatase (LPP); R59949, a DAG kinase inhibitor II; and thimerosal, which inhibits conversion of monoacylglycerol (MAG) to DAG via monoacylglycerol transferase (MGAT). DAG responses were tested for a 15-minute UDP stimulation with and without U73122, U73433, and thimerosal, while studies on R59949 and Propranolol were conducted for the full time course.

As anticipated, U73122 suppressed the UDP response at the 15 min time point for most of the DAG species (Fig. S1), while R59949 and propranolol showed a profound effect on the UDP response over the 30-minute time course. For most DAG species, U73433, the inactive analog of U73122, does not have a significant effect on the UDP response (not shown). Thimerosal shows slight inhibition in the UDP response for the higher carbon number and more unsaturated fatty acid-containing DAG species, suggesting multiple sources of production of these species (Fig. S2).

The R59949 data suggest a dependence on DAG kinase activity in the UDP pathway for both long chain PUFAs and mono- and di-unsaturated DAGs, especially at the early time points (Fig. S3; di-unsaturated DAG data not shown, though qualitatively similar to response of mono-unsaturated DAGs). For most of the species displaying large responses, the increase with UDP + R59949 from the 3 min to 15 min time point was not as steep as with UDP alone, suggesting a combined role for DAG kinase and LPP at the later time points.

Although propranolol had no effect on the UDP response for 38:4 DAG, it eliminated the UDP response in many of the mono- and di-unsaturated DAGs (Fig. S4; di-unsaturated DAG data not shown). Unstimulated cells pretreated with propranolol exhibited an increase in levels of mono- and di-unsaturated and saturated PAs throughout the tested time course (not shown). In stimulated cells pretreated with propranolol, however, there was no significant increase in the mono- and di-unsaturated and saturated PAs over the amount of PA produced from propranolol alone. Propranolol did not significantly increase PUFA-containing PA levels in resting cells and had no significant effect on the UDP response.

Discussion

A mathematical model of the UDP signaling pathway in RAW 264.7 macrophages has been developed to better understand the underlying mechanisms of species-specific DAG production and degradation, as DAG species have been shown to exhibit differential responses to a single stimulus and have also been linked to different cellular processes downstream of receptor activation. To capture the necessary elements and behaviors of this complex signaling pathway while also maintaining a certain degree of simplicity, a minimalistic yet realistic approach has been taken, and a successful quantitative representation of the events downstream of activation of the P2Y₆ receptor has been achieved.

Initial efforts using the canonical pathway for species-specific DAG production and degradation (via PIP₂ hydrolysis only) were unsuccessful, as we were unable to reproduce the behavior seen experimentally from different species of DAG. Specifically, the biphasic dynamics of mono- and di-unsaturated DAGs could not be reproduced for any choice of parameter values. Furthermore, the hypothesis that later DAG production originates from PLD activity does not hold in RAW 264.7 macrophages stimulated with UDP, as we found there to be no PLD activity in these cells upon stimulation with UDP. Instead, upon incorporating into this canonical pathway a known pathway in DAG metabolism involving a second pool of DAG present in high concentrations in resting cells, we were able to obtain *in silico* results comparable to those seen empirically. Under this two-pool model structure for DAG kinetics shown in Fig. 2, we were able to reproduce differential kinetics among multiple species of DAG, holding all model parameters fixed in all equations, where each DAG species was modeled by two equations of the form of Eqs. (7) and (8), where the only differences between DAG species equations were fluctuations in parameters directly related to the production and degradation of the different DAG species. For the same choice of all rate parameters, we obtained a good fit for the experimental results of IP₃ and Ca²⁺. The experimental results for measuring PIP₂ at the same timepoints as DAG measurements during UDP stimulation resulted in no statistically significant differences in their concentrations. Therefore they were not included in the models parameters.

To derive the model equations, the mechanisms of receptor activation and desensitization were modified for the P2Y₆ receptor from a detailed description given by Lemon et al. in P2Y₂ receptors (2003) to accurately represent the slow receptor internalization and desensitization of surface P2Y₆ receptors seen by Brinson and Harden (2001). There are multiple pathways which could be involved in the production of IP₃ and DAG, as multiple G protein subunits and isoforms can activate multiple isoforms of PLC. This often depends on the cell type under

consideration as well as the receptor or receptors undergoing stimulation. Since P2Y₆ receptors have been shown to couple to the Gα_q isoform (Yoshioka et al., 2001), and PLCβ-3 isoforms have been shown to exhibit strongest selectivity to Gα_q (Sternweis and Smrcka, 2007), we have assumed a single mechanism for PIP₂ hydrolysis: activation of Gα_q and PLCβ-3.

As modeling Ca²⁺ dynamics has not been a focus of this work, Ca²⁺ contribution in the signaling pathway has been based on a model previously developed by Li and Rinzel (1994) and has been handled in such a way as to treat Ca²⁺ more as an input into the model. Therefore, parameter values for the Ca²⁺ module were chosen so as to most accurately mimic the experimental Ca²⁺ data in the RAW 264.7 population data from the Alliance for Cellular Signaling (2008).

Since mathematical modeling of this signaling pathway has led to key insights into the mechanisms involved in species-specific DAG production and degradation, the next step is to test these predictions experimentally. We have begun to inhibit specific enzymes in the proposed pathway using pharmacological agents, and the data are in good agreement with predictions of the model. For instance, if PIP₂ hydrolysis is the only other means of DAG production at early time points, the immediate increase in DAGs observed in the presence of R59949 (a diacylglycerol kinase inhibitor) is a result of PIP₂ hydrolysis. The absence of this early rise in DAGs is due to rapid phosphorylation of resting DAGs via DAG kinase activity, as predicted by the two-pool model. The observed increase in saturated, mono-unsaturated, and di-unsaturated PAs in cells pretreated with propranolol suggests there exists a constitutively active DAG kinase in resting cells that is phosphorylating mono- and di-unsaturated and saturated DAGs, as well as a constitutively active LPP that is dephosphorylating these PAs. This also agrees with the two-pool model (Fig. 2), if we assume that propranolol is selectively affecting the part of the pathway involving the contribution of PA within the cell to the resynthesis of PIP₂ at the plasma membrane. Indeed, if there exist high resting levels of mono- and di-unsaturated and saturated DAGs, inhibition of constitutively active LPP leads to a depletion of the DAG pool and an increase in PA. If the remaining parts of the pathway are left intact, upon stimulation we should observe an increase in these DAG species via PA conversion to CDP-DG to PI. This PI is then shuttled to the plasma membrane, phosphorylated to PIP and again to PIP₂, which is hydrolyzed to form DAG. Since we do not see an increase in mono- and di-unsaturated and saturated DAGs with propranolol and UDP, we conclude that propranolol is affecting only this portion of the pathway.

Sensitivity analysis of model output to variations in model parameters was performed using the Latin Hypercube sampling technique in combination with standardized regression coefficients obtained from a multiple linear regression model. The vast majority of multiple regression model slopes, R_y^2 , were all sufficiently close to one for all time points and for all model outputs, with the smallest values being greater than 0.8. Thus, the standardized regression coefficients were proven to be appropriate measures of parameter sensitivity. Both IP₃ and DAG were shown to be sensitive to the rate of receptor phosphorylation, k_p , and the rate of PIP₂ replenishment, k_{rep} , suggesting that the uncertainty in the output of these two variables could be significantly reduced by obtaining a better estimate of these two rate parameters. DAG was most sensitive to the activation and degradation parameters of DAG_{p2} and the degradation rate of DAG_{p1}, which were all directly estimated from the experimental data. To complement the sensitivity analysis presented here, a rigorous qualitative analysis can be found in Callender (2007) and Callender and Horn (2009). In particular, Callender and Horn (2009) describes the global existence, uniqueness, positivity, and boundedness of solutions, and global stability of the steady-state solution of a corresponding reduced system.

Further mathematical treatment involves investigation of spatial aspects of the UDP signaling pathway by incorporation of partial differential equations as well as inclusion of delay terms to better represent what is seen experimentally and known to be true biologically. Modeling is an iterative process. Thus as more experimental results are obtained, we will expand and build upon the current model accordingly, gradually increasing the complexity as well as its ability to provide a good representation of the UDP signaling pathway. Ongoing experimental efforts will further verify the two-pool model by development and testing of gene knockdowns of the enzymes believed to participate in the proposed pathway, with a specific focus on isoforms of DAG kinase.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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(C)

Figure 1.

Timecourse of stimulation with 25 μM UDP in RAW 264.7 cells. Solid black lines represent model simulations for the system of equations using the two-pool model (with Eq. (7) and Eq. (8)), whereas gray lines represent simulations using the canonical pathway for DAG (Eq. (6)). (A) 38:4 DAG response (representative of the response of most polyunsaturated fatty acid-containing DAG species). (B) 34:1 DAG response (representative of the response of most mono- and di-unsaturated fatty acid-containing DAG species). Data points in (A) and (B) contain nine replicates performed on three different experimental days, with error bars = mean $\pm$ 1 SE. Units are total change in ng over baseline levels in $\sim 8 \times 10^6$ cells. (C) IP₃ response in pmols per $\sim 4.5 \times 10^5$ cells. Points represent the average of four experiments, and error bars = mean $\pm$ 1 SE. (D) Ca²⁺ response in μM . Red curve is a representative Ca²⁺ trace taken from the UDP experiments within the AfCS single ligand screen as described in the “Intracellular free calcium assay” portion of the Methods section.

Figure 2.

Proposed two-pool model for DAG kinetics post-agonist stimulation with UDP. Initial production of a single species of DAG from the hydrolysis of PIP₂ in pool 1 (plasma membrane) is offset by phosphorylation of DAG by a DAG kinase in pool 2 (which we conjecture to be the Endoplasmic Reticulum) to aid in the replacement of PI. The second wave of DAG is a result of resynthesis of PIP₂, which is then hydrolyzed to form DAG and IP₃. While production of each species of DAG will follow this diagram, experimental data suggests that rates of production, degradation and transition between the pools vary between species. Abbreviations not mentioned in the text are as follows: PI4K, phosphatidylinositol 4-kinase; PI5K, phosphatidylinositol 5-kinase; DGK, DAG kinase; LPP, lipid phosphate phosphatase; IP₄, inositol 4-phosphate; IP₂, inositol diphosphate; IP, inositol phosphate; MAG, monoacylglycerol; TAG, triacylglycerol; GPL, glycerophospholipid; CDS, CDP-DAG synthase; PIS, phosphatidylinositol synthase; Ins, inositol.

Figure 3.

Model simulations for timecourse of stimulation with 25 μM UDP in a single RAW 264.7 cell for remaining model variables. (A) Activated (solid line) and inactivated (dashed line) P2Y_6 surface receptors. (B) Total number of activated $\text{G}\alpha\cdot\text{GTP}$. (C) Total number of PIP_2 available for hydrolysis.

Figure 4.

Standardized Regression Coefficients (SRCs) corresponding to most sensitive parameters for IP₃. Most sensitive parameters are as follows: receptor rate of phosphorylation, k_p (■), receptor recycling rate, k_r (■), PIP₂ replenishment rate, k_{rep} (■), and IP₃ degradation rate, k_{d3} (■). Inset: R_y^2 values for all time points are ≥ 0.8 , ensuring that the SRCs for IP₃ are good measures of sensitivity.

Figure 5.

Standardized Regression Coefficients (SRCs) corresponding to most sensitive parameters for DAG. Most sensitive parameters are as follows: degradation rate of DAG_{p1}, k_{dp1} (■), production rate of DAG_{p2}, k_{ap2} (■), degradation rate of DAG_{p2}, k_{dp2} (■), receptor rate of phosphorylation, k_p (■), and receptor recycling rate, k_r (■). Inset: Since R_y^2 values for all time points are ≥ 0.95 , the SRCs for DAG are good measures of sensitivity.

Figure 6.

Changes in 34:1 DAG response to 50% variations in (A) the rate of degradation of pool two 34:1 DAG, k_{dp2} , (B) the rate of activation of pool two 34:1 DAG, k_{ap2} , and (C) the rate of degradation of pool one 34:1 DAG, k_{dp1} , from their nominal values of $1.3 \times 10^{-3} s^{-1}$, $3.74 \times 10^{-3} s^{-1}$ and $1.73 \times 10^{-3} s^{-1}$, respectively.

Table 1

Time-independent model variables

Parameter	Value	Reference
[L]	25 μM	
N_{RT}	20000	(Garrad et al., 1998; Mahama and Linderman, 1994)
K_1	5 μM	(Lemon et al., 2003; Lukas, 2004)
K_2	100 μM	(Lemon et al., 2003)
k_r	$1.75 \times 10^{-4} \text{ s}^{-1}$	(Lemon et al., 2003)
k_p	$2.65 \times 10^{-3} \text{ s}^{-1}$	see text
k_e	$6 \times 10^{-3} \text{ s}^{-1}$	(Lemon et al., 2003)
N_{GT}	3×10^5	(Flaherty, 2007)
k_a	0 137 $\mu\text{M}^{-1} \text{ s}^{-1}$	(Flaherty, 2007)
k_d	0.02 s^{-1}	(Mahama and Linderman, 1994)
N_{PIP_2T}	9×10^6	see text
v	$5 \times 10^{-13} \text{ L}$	(Sasada et al., 1983)
K_C	0.1 μM	see text
$\hat{k}_{hyd}$	$1.89 \times 10^{-6} \text{ \#mol}^{-1} \text{ s}^{-1}$	see text
k_{rep}	$7.04 \times 10^{-3} \text{ s}^{-1}$	see text
k_{d3}	$4.5 \times 10^{-3} \text{ s}^{-1}$	see text
N_{DAGp_2T}	480 μM (500 μM) ^a	see text
k_{dp1}	$7 \times 10^{-3} \text{ s}^{-1}$ ($1.73 \times 10^{-3} \text{ s}^{-1}$) ^a	see text
k_{dp2}	$3.7 \times 10^{-3} \text{ s}^{-1}$ ($1.3 \times 10^{-3} \text{ s}^{-1}$) ^a	see text
k_{ap2}	$1.08 \times 10^{-2} \text{ s}^{-1}$ ($3.74 \times 10^{-3} \text{ s}^{-1}$) ^a	see text
η	0.66 (0.45) ^a	see text
ε	0.01	(De Young and Keizer, 1992; Li and Rinzel, 1994)
d_{IP_3}	0.053 μM	see text
d_{act}	0.4 μM	(Li and Rinzel, 1994)
d_{inh}	0.4 μM	(Li and Rinzel, 1994)
α	4 s^{-1}	(Li and Rinzel, 1994)
v_1	40 s^{-1}	(Li and Rinzel, 1994)
v_2	0.02 s^{-1}	(Li and Rinzel, 1994)
v_3	2 $\mu\text{M} \text{ s}^{-1}$	see text
v_4	1.8 $\mu\text{M} \text{ s}^{-1}$	(Li and Rinzel, 1994)
k_{er}	0.2287 μM	see text
k_{pl}	0.1 μM	(Li and Rinzel, 1994)
j_{in}	0.04 $\mu\text{M} \text{ s}^{-1}$	see text
c_1	0.185	(De Young and Keizer, 1992)

^aParameter values not in parentheses correspond to 38:4 DAG, whereas those in parentheses are for 34:1 DAG.