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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*
PLCγ1 hydrolyzes the lipid substrate PIP_2 to the two second messengers, IP_3 and DAG, in response to certain growth factors, such as EGF. PLCγ1 is often overexpressed and activated in breast tumors. Overexpression and activation of PLCγ1 in breast tumors have been correlated with increased cell motility, therefore, PLCγ1 may play a role in tumor metastasis. As a consequence, PLCγ1 is an attractive target for novel anti-cancer therapies. My laboratory proposes to investigate if a computer simulation program can be generated, based on existing experimental data, that can predict the kinetic behavior of PLCγ1. Creation of an kinetic simulation program for PLCγ1 will allow us to predict which steps are rate-limiting and then design experiments to test these predictions. The new experimental data will then be used to refine the simulation program. This approach, if successful, will allow a more rapid identification of potential drug targets. Additional benefit of the program is that it may be able to describe the kinetic behavior of any phospholipase or enzyme, which has been implicated in the progression of breast cancer, that requires binding to the membrane in order to be active.

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Introduction

The inositol signaling enzyme, phospholipase C-γ1, (PLC−γ1), catalyses the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), to the two second messengers, inositol 3,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). These two second messenger play important roles in the regulation of cellular function. Abnormal levels of DAG have been associated with tumor formation and growth. Overexpressed and activated of PLCγ1 has been seen in breast cancer tumors (1, 2). One of the results of overexpression and activation of PLCγ1 in breast tumors appears to be increased cell migration. Therefore, it has been proposed that PLCγ1 may play a role in tumor metastasis (3). Inhibition of PLCγ1 function by various agents has been shown to prevent growth factor-induced cell migration (3), which makes this enzyme an attractive target for novel anti-cancer therapies. Since PLCγ1 also plays a role in normal cellular function, the global inhibition of this enzyme is not desirable. Also, PLCγ1 is one member of a family of isozymes that catalyze the hydrolysis of PIP_2. The other isozymes have been implicated in playing roles in the cell different from PLCγ1. However, all of the iosyzmes in this family are believed to have the same catalytic mechanism, only the modes of regulation are different. Any catalytic inhibitor of PLCγ1 will also inhibit the other isozymes. Therefore, agents designed to inhibit the regulation of PLCγ1 would be ideal. The purpose of this Concept grant was two-fold; 1) to investigate if kinetic simulation programs based on the current kinetic model for the hydrolysis of PIP_2 by PLCγ1 using existing experimental data can simulate the kinetic behavior of PLCγ1 and 2) to use the kinetic simulation program to identify constriction points for the production of PIP_2.

Body

Statement of Problem

In response to EGF, PLCγ1 undergoes translocation from the cytosol to the membrane and becomes activated. Though calcium is required for activity, the translocation process does not appear to calcium dependent. Review of the literature indicates that levels of certain anionic lipids maybe the regulator of the membrane binding step. In addition to be possible regulators of membrane binding, anionic lipids also appear to regulate the production of IP_3.

It is not clear how these calcium or anionic lipids regulate PLCγ1 activity nor is it clear which reaction steps are being regulated by these agents. The first step in being able to design agents to regulate the amount of PIP_2 hydrolyzed by PLCγ1 is to be able to identify the steps that control the flux through the pathway. Using current experimental systems, it is not possible to determine if which steps in PIP_2 hydrolysis by PLCγ1 are responsible for controlling flux through the system

Background and Significance

The current kinetic model for PIP_2 hydrolysis by PLCγ1 was proposed based on *in vitro* kinetic experiments (Fig 1) (4). In this model cytosolically located PLCγ1 (E) first binds to the membrane (M) to form the enzyme bound complex (EM) (step 1). Once on the membrane surface, substrate (S) can diffuse into the active site after which hydrolysis of PIP_2 occurs (step 2). Successive cycles of diffusion of PIP_2 into the active site with subsequent PIP_2 hydrolysis can occur before the enzyme dissociates from the membrane

surface. This type of reaction can be regulated at either step 1, membrane binding or step 2, substrate catalysis. Experiments conducted so far has demonstrated little regulation of step 1, which is consistent with the literature.

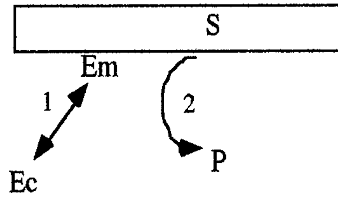
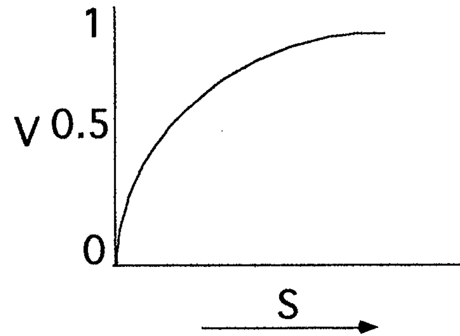


Figure 1. Model of production of IP_3 by PLCγ1. Step 1; Cytosolic PLCγ1 (Ec) associates with the membrane to form the enzyme:membrane complex (EM). Step 2; The enzyme:membrane complex (EM) hydrolysis PIP_2 (S) to IP_3 (P).

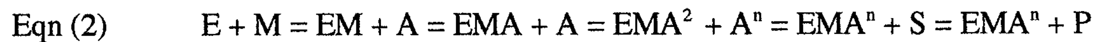
In the simplest form, this reaction would follow an order, Bi Bi kinetic mechanism. Since the membrane is assume to be saturating, this reaction would simplify to Michaelis-Menton reaction mechanism, generating a hyperbolic velocity curve in response to increasing substrate.

Eqn (1)

$$E + M = EM + S \longrightarrow EM + P$$



However, experimental data measuring the amount of IP_3 formed by PLCγ1 as a function of PIP_2 concentration generated sigmodal curves (4). Indicating that catalysis of PIP_2 by PLCγ1 follows a mechanism that involves binding of an activator (A) to a site separate from the active site. This suggests that step 2, the hydrolysis of substrate, is the regulated step. Further support for the regulation of step 2 by an activator was obtained when it was shown that other anionic lipids, lipids that can not be hydrolyzed by PLCγ1, could increase PIP_2 hydrolysis. In the presence of the anionic lipids, the reaction kinetics switched from a sigmodal function to hyperbolic function. The parameter affected appeared to be substrate binding, in the presence of activator the half-maximal binding of PLCγ1 decreased 10-fold. The change in the kinetic behavior of PLCγ1 in the presence of the lipid activator suggests that the enzyme exists in at least two conformations on the membrane surface, inactive and active. As a result of these experiments equation 1 was written to include an inactive and active form of the PLCγ1 on the membrane surface.

Eqn (2) 

$$E + M = EM + A = EMA + A = EMA^2 + A^n = EMA^n + S = EMA^n + P$$

However, these kinetic experiments did not directly measure the membrane binding step. Experiments designed to measure the membrane binding step, found that membrane composition did not affect membrane binding, but did affect calcium binding.

Data showed that PLCγ1 had two single ion binding sites for calcium (Miller and Jones, unpublished results). Site 1 had an low affinity for calcium (0.02 μM) and is presumed to be the active site, Site 2 was found to have an variable affinity for calcium. In solution, the calcium affinity was 10 μM, in the presence of neutral phospholipids the affinity was 1 μM and in the presence of activating lipids the affinity was 0.04 μM (Jones, unpublished results). These data gave experimental evidence to the conclusions from the kinetic experiments that there are at least two conformations of membrane PLCγ1, depending on the presence of activating lipid. However, these experiments can not allow us to conclude if the change in calcium affinity in the presence of activating lipid will effect the production of IP_3.

Method

Metabolic control analysis (MCA) is designed to aid in the solving of these type of problems. Using user defined parameters, MCA will allow the determination of the flux through a system at steady-state. Analysis of perturbations at various steps will allow the determination of which steps are responsible for controlling the flux through the system. Currently an number of freeware programs are available. In the proposal, simulations using the current kinetic mechanism and experimental derived kinetic parameters for the hydrolysis of PIP_2 by PLCγ1 were run using the freeware Gepasi. From these simulations the followed questions were asked:

- Do the simulations resemble experimental obtained results?
- What step most influence the amount of PIP_2 hydrolyzed ?

Results

For the initial simulations, each reaction step in the hydrolysis of PIP_2 by PLCγ1 was entered in MCA program. All competing reaction steps were included, This situation likely mimics the actual events (Fig. 2). The kinetic parameters used were those that had be previously determined experimentally (Table 1). A kinetic mechanism based on the experimental results was selected for each step. The results of these simulations did not mimic the experimental data. Further analysis revealed that using the reaction scheme as written, the program could not determine a steady-state for the reaction scheme. Breaking the reaction scheme into sub-reactions revealed that the problem appeared to lie with formation of product from substrate. Attempts to solve this problem, have so far been unsuccessful. It is possible that kinetic mechanisms propose for each step in the reaction scheme is incorrect. Selecting different mechanisms did not solve the problem. It is also possible the kinetic parameters derived are incorrect. The MCA program contains functions that will allow the determination of kinetic parameters from the fitting of experimental data. Currently, all previously collected experimental data is being converted into a format that can be read by the program. Once completed, the data will be fitted using the MCA program. It is also possible that more reaction steps are required for the hydrolysis of PIP_2 than have been proposed. If this is the case addition experiments will need to be conducted.

Table 1. Kinetic mechanisms and parameters used for reaction scheme depicted in Fig. 2.

Reaction	Kinetic Mechanism	Kinetic parameters
E <-> Eca	Mass action	K_s= 4 μM (Jones, unpublished results)
Eca <-> EMCa	Mass action	K_s = 250 μM (Jones, unpublished results)
EMCa<-> ESCa	Catalytic activator	K_m = 0.2 mf, K_s = 1 mM (4)
E <-> ExM	Mass action	K_s = 250 μM (Jones, unpublished results)
ExM <-> ExMCa	Mass action	K_s = 1 μM (Jones, unpublished results)
ExMCa <-> ExACa	Catalytic activator	K_m = 0.2 mf, Ks = 100 μM (4)
ExM <-> ExA	Hyperbolic modifier	K_s = 100 μM (4)
ExA <-> ExAxCa	Mass action	K_s = 0.4 μM (Jones, unpublished results)
S <-> P	Explicit Michaelis- Menton	K_m = 0.05 mf (4)

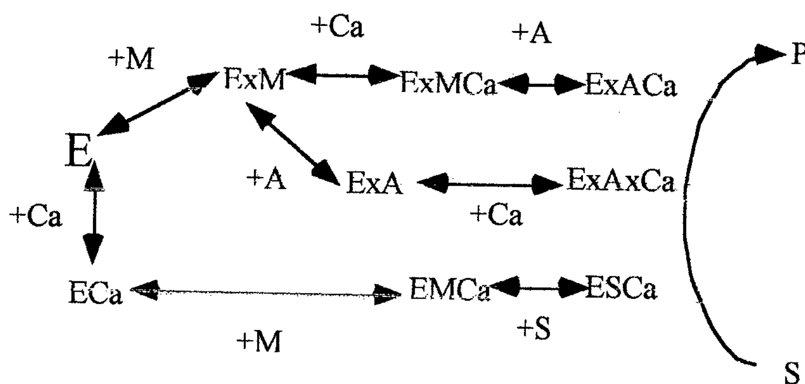


Fig 2. Possible reaction scheme describing the formation of IP_3 by PLCγ1. Starting with PLCγ1 located in the cytosol (E), depicted here are possible reaction schemes for the formation of IP_3. This reaction schemes are based on the available experimental data. PLCγ1 (E); membrane (M); calcium (Ca); A (anionic lipid activator); PIP_2 (S); IP_3 (P).

It was possible to model the membrane binding steps of the reaction scheme (Figure 3). The simulations run mimicked data that was obtained experimentally. Following is an examples of the type of questions that can be answered from these type of simulation studies. In the cell, activation of enzyme cascades by calcium occur around 0.3 μM. From a simulations run under conditions described in Table 2, it can be seen that significant concentrations of active complex are only formed when membrane binding precedes calcium binding to site 2 (>80% for membrane binding first, in the absence of anionic lipids, as compared to < 5% for calcium binding first (Figure 4) and >90% for membrane binding in the presence of activating lipid, as compared to < 1% for calcium binding first (Figure 5)). More interesting is the simulation that demonstrate where half-maximal formation of active enzyme complex occurs (Figure 6 and 7). For

reaction 1, half-maximal formation of active complex occurred at 3-4 μM (EMCa) and for reaction 2, the half-maximal formation of the enzyme complex occurred at 1uM in the absence of activating lipid (Figure 6) and 0.3 μM in the presence of activating lipid (Figure 7). The flux due to the formation of active complex was compared for the system (Figure 8 and 9). In the presence of activating lipid, large changes in flux occurred at 0.075 μM and 0.2 μM (Figure 9), whereas in the absence of activating lipid, significant change is the flux occurs at 1 μM (Figure 7). These simulations indicate that in the presence of activating lipid the formation of active PLCγ1 is sensitive to changes in calcium concentration and furthermore, that changes in calcium concentration within the physiological range could have a profound effect on active complex formation. However the problem with these simulations is that under saturating concentrations of membrane, such as would be found in the cell, most of the enzyme would be bound to the membrane. This is not the case in cells. Under resting conditions <20% of PLCγ1 is associated with the membrane (5). The discrepancy between what occurs in the cell and the simulation results obtained from in vitro experiments may indicate that steps are missing in the reaction scheme. One possible missing step is that PLCγ1 prefers to bind to lipid domains (6). Kinetic reactions are being written to test this possibility.

Table 2. Kinetic Mechanism and parameters for the reaction scheme depicted in Fig. 3.

Reaction	Kinetic Mechanism	Kinetic Parameters
E <-> Eca	Mass action	K_s = 4 μM (Jones, unpublished results)
Eca <-> EMCa	Mass action	K_s = 250 μM (Jones, unpublished results)
E <-> ExM	Mass action	K_s = 250 μM (Jones, unpublished results)
ExM <-> ExMCa	Mass action	K_s = 0.4 μM (Jones, unpublished results)

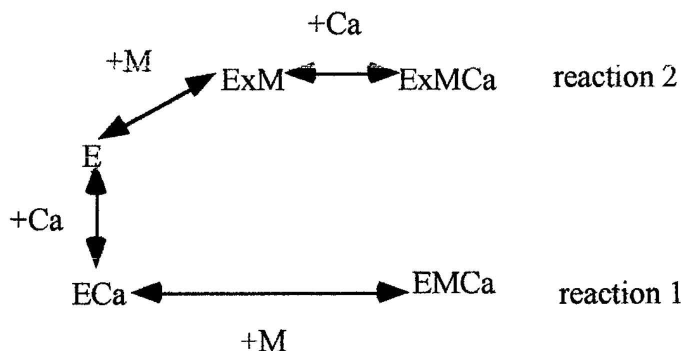


Figure 3. Competing reactions for the formation of the active complex of PLCγ1. The cytosolic located enzyme (E) can either bind calcium (Ca) in the cytosol, then associate with the membrane (M) to form the active complex (EMCa) [reaction 1] or associate with the membrane then bind calcium to form the active complex ExMCa [reaction 2].

These simulations suggest focusing experimental efforts on the binding of anionic lipid to PLCγ1 would be beneficial. The simulations indicate that by inhibiting the anionic lipid induce changes in calcium affinity, would regulate the flux through the system by shifting the formation of active enzyme complex to calcium concentrations greater than that physiologically seen.

In addition to the simulations presented in this report, simulations conducted under the following conditions are ongoing:

1. What effect increases in membrane binding, like observed with tyrosine phosphorylated PLCγ1, affects membrane binding and formation of active complex enzyme.
2. What effect increases in tyrosine phosphorylated enzyme in response to EGF has on membrane binding and formation of active enzyme complex.
3. What effect formation of lipid clusters, such a rafts formed in cells, would have on membrane binding.

To date reactions have been written for all three and simulations run for 1 and 2, but the reactions have not been linked together yet.

Figure 4. Amount of active complex formed in the absence of activating lipid.

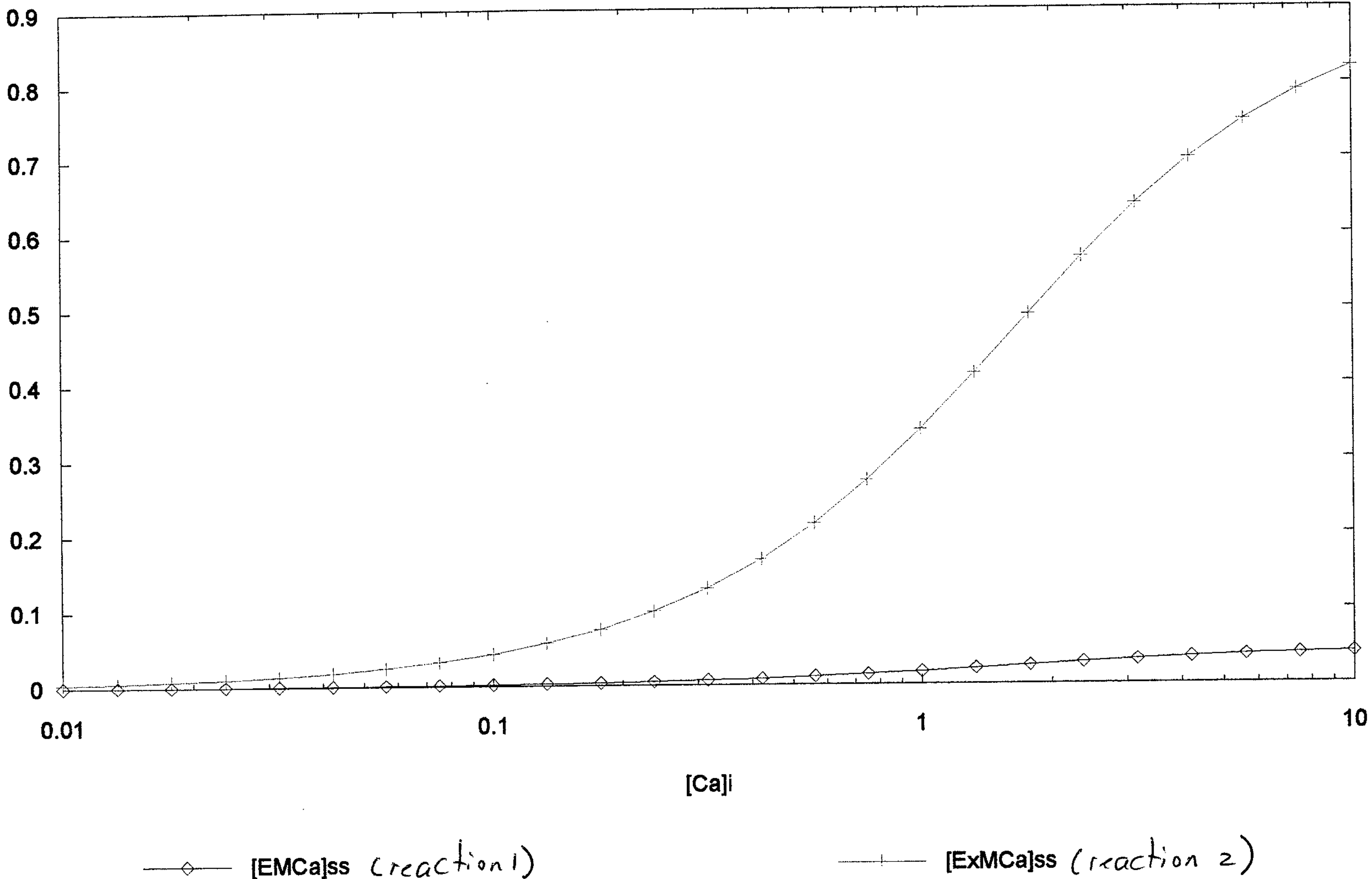


Fig. 4. Amount of active complex formed in the absence of activating lipid. A competing simulation was run based on the reaction scheme depicted in Fig. 3. From the simulation, it was observed that as calcium concentration increased, a large increase in active enzyme complex was formed for reaction 2. Little active enzyme complex was formed through reaction 1.

Figure 5 Amount of active competing enzyme complex formed in the presence of activating lipid.

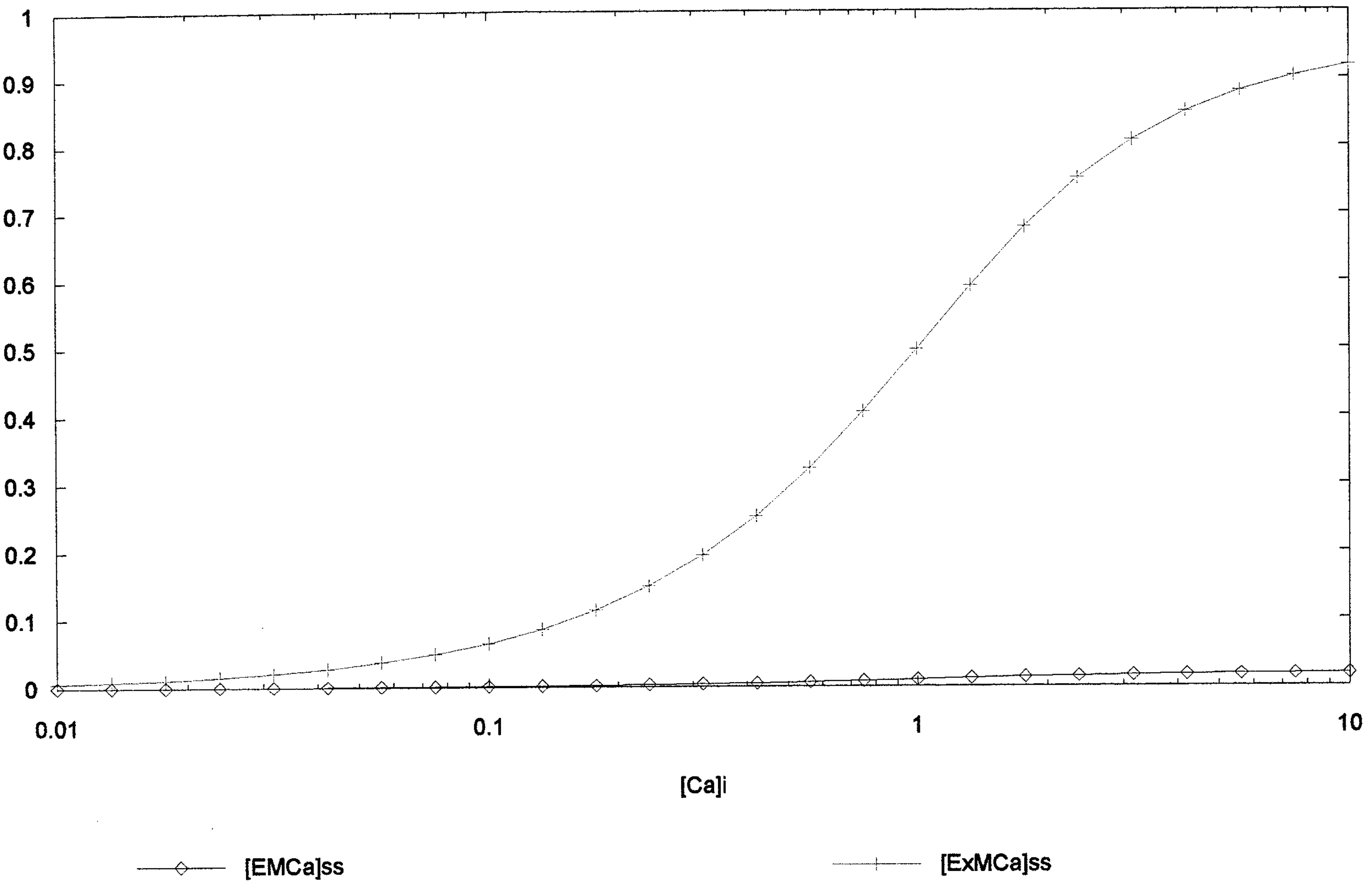


Fig. 5. Amount of active complex formed in the presence of activating lipid
A simulation was run based on the reaction scheme depicted in Fig 3. using kinetic parameters presented in Table 2. From the simulation, it was observed that as calcium concentration increased, a large increase in active enzyme complex was formed for reaction 2. Little active enzyme complex was formed for reaction 1.

Figure 6 Half-maximal formation of active enzyme complex in the absence of activing lipid

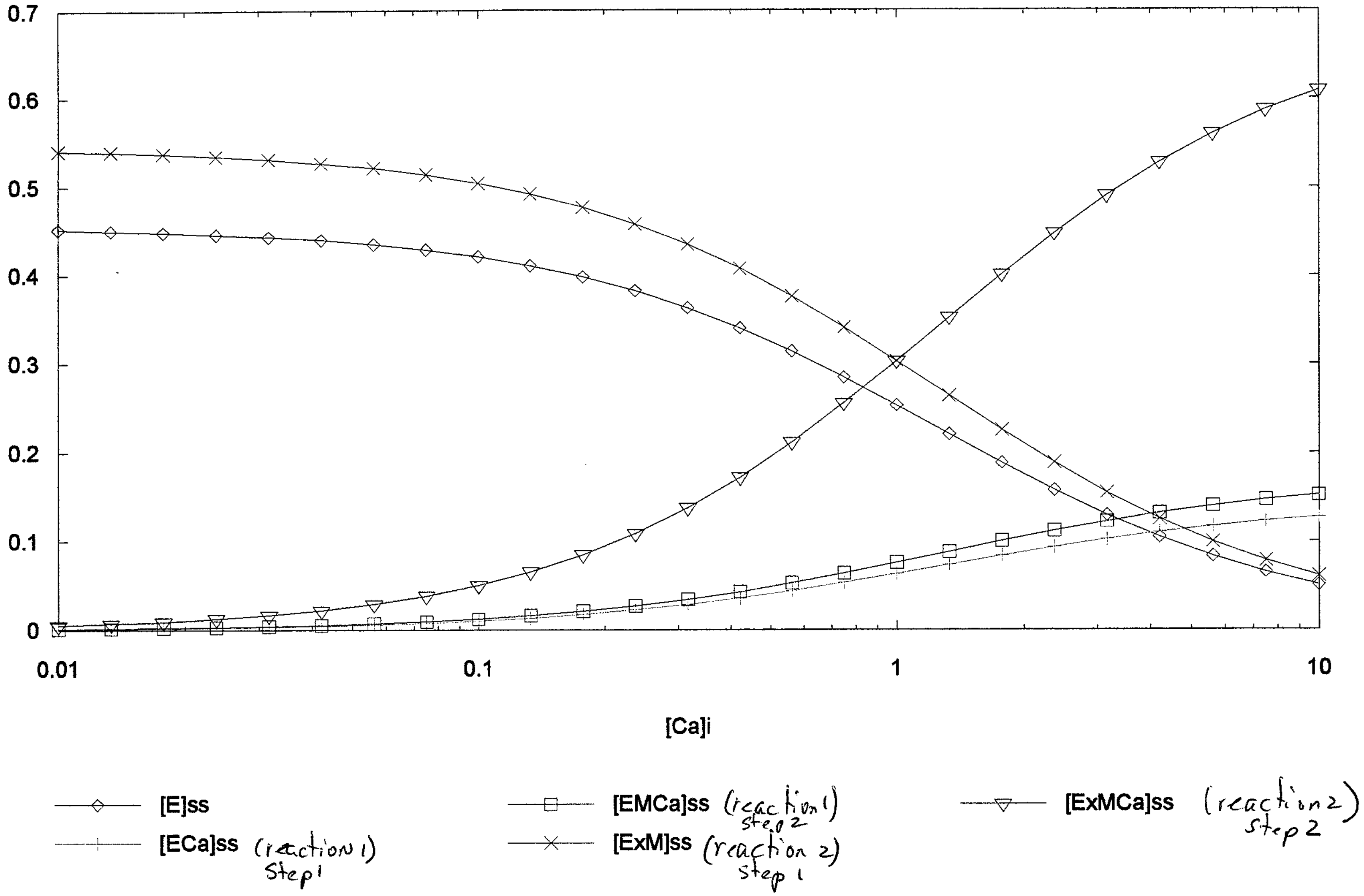


Fig. 6 Half-maximal formation of active enzyme complex.
From the reaction scheme depicted in Fig 3 and using the kinetic parameters presented in Table 2, a simulation was run under condition of varible calcium concentration and no activating lipid. Half-maximal formation of active complex occurred at 4µM for reaction 1 and 1µM for reaction 2.

Figure ~~7~~. Half-maximal formation of active enzym complex in the presence of activating lipid

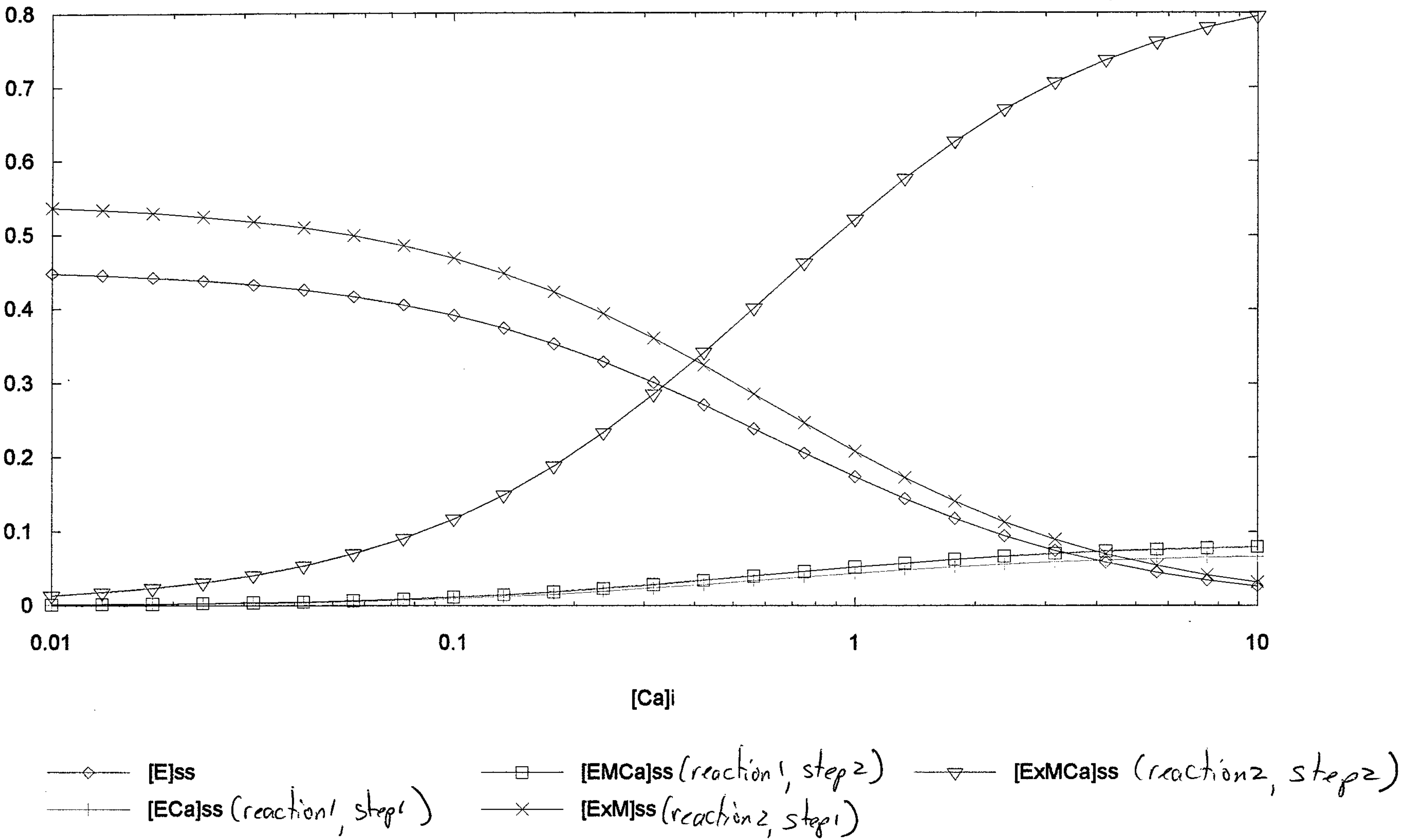


Fig 7. Half-maximal formation of active enzyme complex

From the reaction scheme depicted in Fig 3. and using the kinetic parameters presented in ~~Fig3~~ Table 2, a simulation was run under conditions of varible calcium concentration and no activating lipid. Half-maximal formation of active complex occurred at 3.5uM for reaction 1 and 0.4um for reaction 2.

Figure 8 Flux through the pathway depicting in Fig. 3.

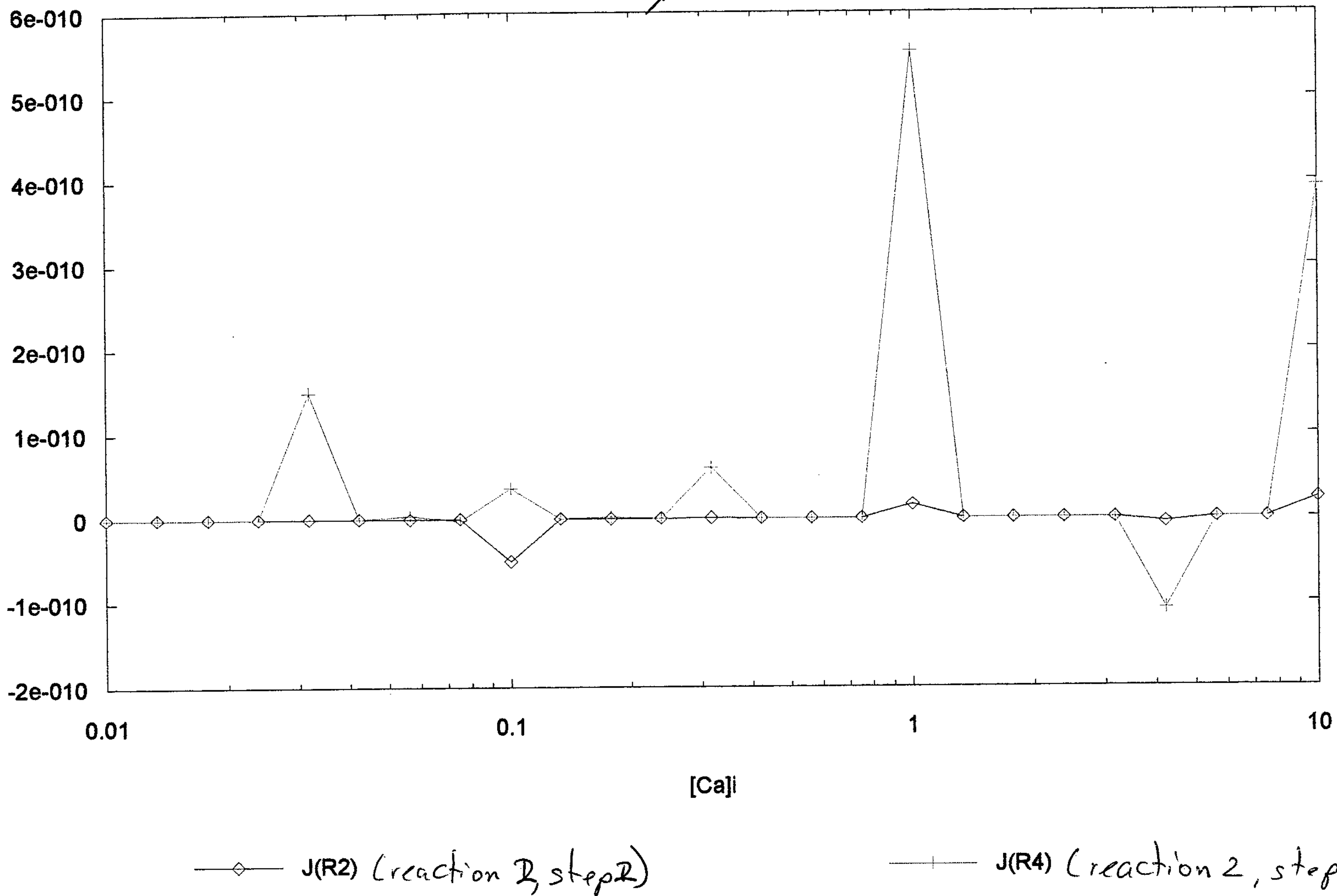


Fig 8. Flux through the pathway depicted in Fig 3.
A simulation was run based on the reaction scheme depicted in Fig 3. Kinetic parameters used were those described in Table 2, in the absence of ~~cat~~ activating lipid. As can see, a major change in flux for reaction 2 occured at 1uM calcium, the same concentration where 50% of the active complex is formed.

Figure 9. Flux through the pathway depicted in Fig 3

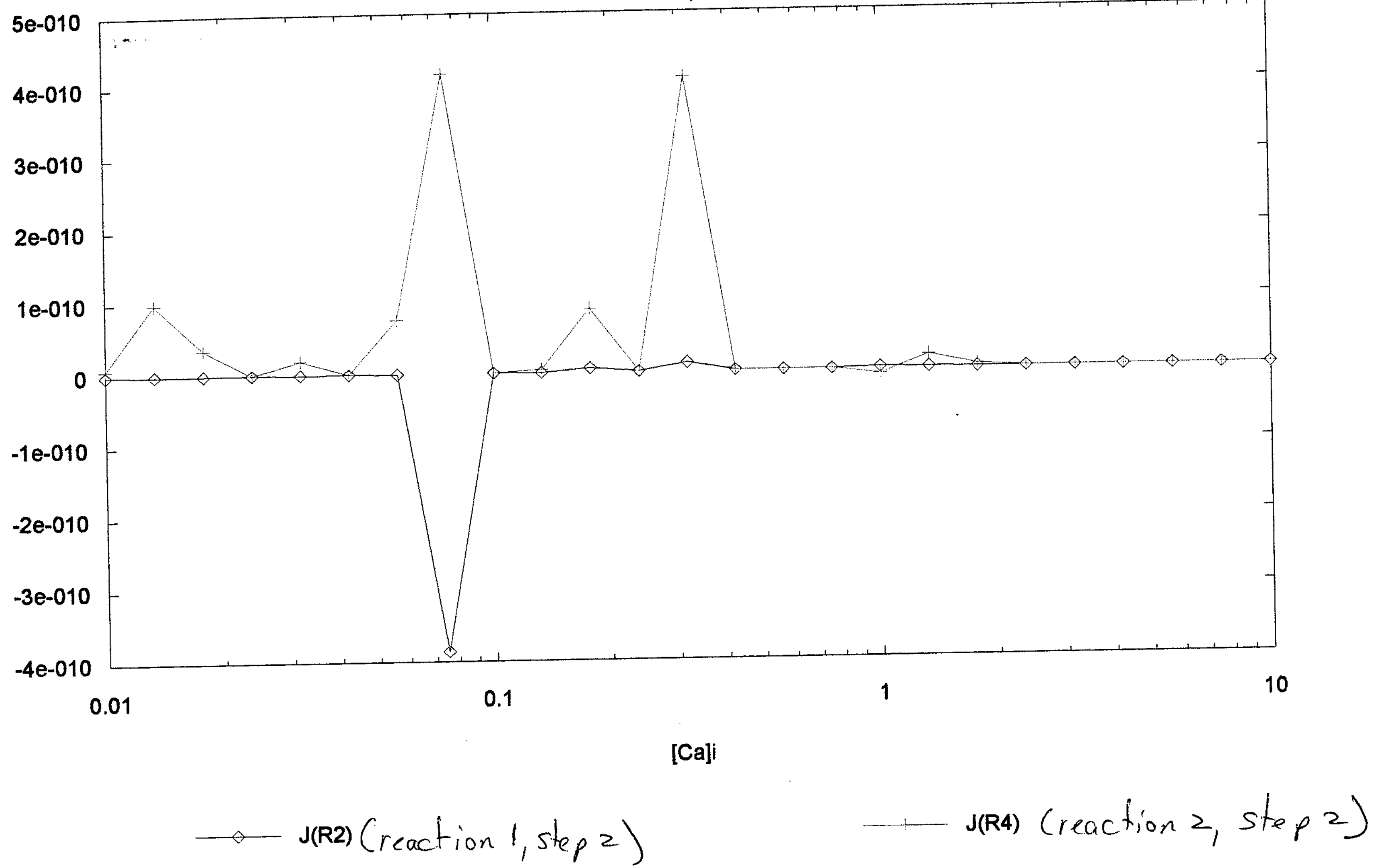


Fig 9. Flux through the pathway.
A simulation was run based on the reaction scheme depicted in Fig 3. Kinetic parameters used were those described in Table 2, in the presence of activating lipid. It was observed that two major changes in flux occurred, one at 0.075 um calcium and the other at 0.2 um calcium.

Key Research Accomplishments

The key research accomplishments for this projected were:

- Various MCA programs were evaluated and one was found that was suitable for the PLCγ1 analysis (Gepasis).
- Reaction equations for the binding the PLCγ1 binding step and enzyme activation were written and evaluated.
- Simulations, using kinetic equations from experimental data, of the PLCγ1 binding and activation step mimicked the experimental results.
- Simulations of the PLCγ1 binding and enzyme activation indicate that membrane binding probably occurs before calcium binding.
- Simulations of the PLCγ1 binding and enzyme activation indicate that the most probable control point for this reaction is the calcium binding step.
- Inhibition of the calcium binding step either by controlling calcium concentration or decreasing the affinity of calcium for PLCγ1, did not affect membrane binding, but did effect formation of the active enzyme.

Reportable Outcomes

To date: none

Conclusions and Future directions

Simulations conducted so far has shown that this approach could be quite useful in the identification of possible regulatory steps involved in the EGF-induced hydrolysis of PIP_2 by PLCγ1. Based on the results of the membrane binding simulations, additional experiments will be conducted to investigate the change in calcium binding affinity in the presence of anionic lipids. Our first attempt to model the reaction scheme from membrane binding to formation of product was not successful. The best approach will be to break down the reaction scheme into subunit that correspond to the experimental data. Once the subunit reactions have been modeled and the results of the simulation resemble the experimental data, then the subunit reactions can be linked together.

The advantages to being able to simulate EGF-induced hydrolysis of PIP_2, is that the simulation will take into account all known perturbations on the system. Experiments conducted to data have looked at only certain parts of the reaction scheme under controlled conditions. Regulatory steps of an isolated system may not be relevant when all perturbations to a reaction are taken into account. The use of MCA may indicate novel regulatory steps that can be targeted for rational drug design. The disadvantage of MCA is that it is a computer simulation and may have no relevancy to what occurs in the cell. It will be important to test the results of the simulation in both isolated controlled systems and in cells.

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