Two roles for Ca^{2+} in agonist stimulated Ca^{2+} oscillations

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ABSTRACT We propose a mechanism for agonist-stimulated Ca^{2+} oscillations that involves two roles for cytosolic Ca^{2+}: (*a*) inhibition of inositol-1,4,5-trisphosphate (IP_3) stimulated Ca^{2+} release from the endoplasmic reticulum (ER) and (*b*) stimulation of the production of IP_3 through its action on phospholipase C (PLC), via a G_q protein related mechanism. Relying on quantitative experiments by Parker, I., and I. Ivorra (1990. *Proc. Natl. Acad. Sci. USA.* 87:260–264) on the inhibition of Ca^{2+} release from the ER using caged-IP_3, we develop a kinetic model of inhibition that allows us to simulate closely their experiments. The model assumes that the ER IP_3 receptor is a tetramer of independent subunits that can bind both Ca^{2+} and IP_3. Upon incorporation of the action of Ca^{2+} on PLC that leads to production of IP_3, we observe in-phase oscillations of Ca^{2+} and IP_3 at intermediate values of agonist stimulation. The oscillations occur on a time scale of 10–20 s, which is comparable to the time scale for inhibition in *Xenopus* oocytes. Analysis of the mechanism shows that Ca^{2+}-inhibition of IP_3-stimulated Ca^{2+} release from the ER is an essential step in the mechanism. We also find that the effect of Ca^{2+} on PLC can lead to an indirect increase of cytosolic Ca^{2+}, superficially resembling "Ca^{2+}-induced Ca^{2+}-release." The mechanism that we propose appears to be consistent with recent experiments on REF52 cells by Harootunian, A. T., J. P. Y. Kao, S. Paranjape, and R. Y. Tsien. (1991. *Science* [*Wash. DC*]. 251:75–78.) and we propose additional experiments to help test its underlying assumptions.

INTRODUCTION

Oscillations in cellular Ca^{2+} concentrations are a widespread phenomenon that have been investigated intensely using both experimental and theoretical methods. The period of these oscillations, which have been observed electrophysiologically and with fluorescent dyes, varies from cell type to cell type and ranges from a few seconds to the order of 10 min (Berridge, 1989, Berridge and Galione, 1988). In some cells, Ca^{2+} oscillations can be stimulated by exogenous hormones or other agonists and appear to be independent of external Ca^{2+}, whereas in other cells oscillations seem to require external Ca^{2+} and to be independent of agonist-receptor coupling (Grapengiesser et al., 1989). Thus, despite the superficial similarities in the appearance of Ca^{2+} oscillations, it seems likely that they may arise from different mechanisms in different cell types.

For oscillations that are agonist-stimulated, it is widely believed that release of Ca^{2+} from the endoplasmic reticulum (ER) is a crucial part of the mechanism. Over the past few years abundant evidence has accumulated that this is controlled by the binding of inositol-1,4,5-trisphosphate (IP_3) to membrane-bound receptors in the ER (Berridge and Irvine, 1989). Whereas other phospholipid metabolites are also implicated in the release of Ca^{2+} from internal stores (Turk et al., 1987), IP_3-stimulated release from the ER appears to play a central role and is thought to be important in the mechanism of Ca^{2+} oscillations in a number of cell types (Berridge and Galione, 1988).

A variety of different mechanisms, each focusing on particular aspects of Ca^{2+} handling, have been proposed to explain Ca^{2+} oscillations (Berridge, 1989, Harootunian et al., 1991). Lately, several of these have been refined into detailed mathematical models. Following the suggestions of Fabiato and others (Fabiato and Fabiato, 1975, Kuba and Takeshita, 1981), Goldbeter and colleagues (Dupont and Goldbeter, 1989, Dupont et al., 1991) have proposed a model based on the idea that elevated cytosolic Ca^{2+} can induce Ca^{2+} release from an intracellular store. In this mechanism, IP_3 stimulates Ca^{2+} release from the ER, but does not itself oscillate. Evidence (Wakui et al., 1989) has been presented that such a mechanism may function in mouse pancreatic acinar cells. Meyer and Stryer, 1988, have proposed another mechanism in which the kinetics of the binding of IP_3 to the ER and subsequent release of Ca^{2+} play a more active role in the oscillations. To actually achieve oscillations with their mechanism, it was necessary to invoke a cooperative uptake of Ca^{2+} by another internal pool, initially identified as the mitochondria. In the Meyer-Stryer model, IP_3 oscillates in phase with cytosolic Ca^{2+}. A variation on this model has been proposed recently by Somogyi and Stucki, 1991. Their model does not require cooperative uptake of Ca^{2+}, but utilizes instead Ca^{2+}-calmodulin stimulation of IP_3 sensitive Ca^{2+}

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channels in the ER. In this model, however, IP_3 does not oscillate.

Another class of models has been introduced by Cuthbertson and Chay, 1991. They focus on the postulated nonlinear kinetics associated with the agonist-receptor mechanism, including interactions among G-proteins and the dynamics of membrane bound diacylglycerol and protein kinase C. Finally the model of Swillens and Mercan, 1990, which involves a single IP_3-sensitive Ca^{2+} store, invokes negative feedback of Ca^{2+} from within the ER on the IP_3 channels of the ER. This is similar, mathematically, to the Somogyi-Stucki model in that negative feedback of ER Ca^{2+} has the same effect as stimulation of the IP_3-sensitive channel by cytosolic Ca^{2+}.

Many of these models were conceived before the discovery that cytosolic Ca^{2+} actually inhibits the release of Ca^{2+} through IP_3 channels in the ER. Quantitative experiments by Parker and Ivorra, 1990*a*, using caged IP_3 have shown that inhibition by Ca^{2+} occurs on the time scale of 1–20 s, suggesting that it may comprise an important negative feedback step for oscillations with periods of the order of a minute or less. In this paper we propose a kinetic mechanism to explain the observations of Ca^{2+} inhibition of IP_3-stimulated Ca^{2+} release from the ER. Separate kinetic and equilibrium data on the ER IP_3 channel are used to fit the kinetic constants, and we show that the mechanism provides a quantitative explanation of the threshold and inhibition curves obtained by Parker and Ivorra in *Xenopus* oocytes (Parker and Ivorra, 1990*a*, *b*).

The kinetics of IP_3-stimulation and Ca^{2+}-inhibition of Ca^{2+} release from the ER does not of itself lead to oscillations in cytosolic Ca^{2+}. Recently, however, it has been shown that a G-protein (G_q) binds to and activates polyphosphoinositide specific phospholipase C (PLC) (Smrcka et al., 1991). The binding of G_q to PLC greatly enhances the stimulatory effect of Ca^{2+} on its phospholipase activity. It now seems likely that G_q is the G-protein involved in the phospholipid pathway connected with agonist-receptor mediated Ca^{2+} release. Because IP_3 is a product of the action of PLC, this step could provide a direct positive feedback of Ca^{2+} on the production of IP_3 (Smrcka et al., 1991, Taylor and Exton, 1987) to counteract the negative feedback of Ca^{2+} on Ca^{2+} release from the ER and, thus, lead to oscillations in cytosolic Ca^{2+}.

Using our kinetic model of Ca^{2+}-inhibited Ca^{2+} release from the ER, we have investigated this possibility in some detail. Assuming a simple hyperbolic stimulation of IP_3 production with a K_d for Ca^{2+} in the micromolor range, we find that an increase in the fraction of active PLC molecules leads to oscillations in cytosolic Ca^{2+} without the need to modify other kinetic parameters. We provide a brief analysis of the oscillations, which shows that they can be thought of as arising from feedback between Ca^{2+} and the fraction of subunits of the IP_3 Ca^{2+} channel that are in the unbound state.

The mathematical model that results from this mechanism is related to the models of Meyer and Stryer (Meyer and Stryer, 1988, 1991) in the sense that it involves the kinetics of IP_3 stimulated Ca^{2+} release from the ER. It differs from their mechanism and other related mechanisms in two significant features. First, it incorporates a greater level of kinetic detail, including the phenomenon of Ca^{2+} inhibition. And second, it invokes only the positive feedback of Ca^{2+} on agonist-stimulated, PLC-catalyzed production of IP_3. Like the Meyer-Stryer model oscillations in Ca^{2+} are accompanied by an in-phase oscillation of IP_3. Recent experimental results of Harootunian et al., 1991, on Ca^{2+} oscillations in REF52 fibroblasts suggests that IP_3 does oscillate in that agonist-stimulated system. Harootunian et al., 1991, conclude that the dominant feedback mechanism in their preparation appears to be the stimulation of PLC by Ca^{2+}, and we propose that our mechanism may explain agonist-stimulated Ca^{2+} oscillations in REF52 fibroblasts.

Ca^{2+} INHIBITION OF Ca^{2+} RELEASE

Although experiments by Willems et al., 1990 suggest that cytosolic calcium concentrations, $[Ca_i^{2+}]$, in the physiological range (0.1–0.2 μM) do not inhibit IP_3-mediated Ca^{2+} release and that $[Ca_i^{2+}]$ must reach micromolor levels (2–3 μM) before inhibition occurs, there are an increasing number of reports indicating that Ca^{2+} inhibition of Ca^{2+} release may be an important feature in the handling of Ca^{2+} by the ER. Inhibition of Ca^{2+} release by Ca^{2+} has been reported in a number of cells, e.g., adrenal chromaffin (Föhr et al., 1991, Robinson and Burgoyne, 1991), mouse pancreatic acinar cells (Wakui and Petersen, 1990), limulus photoreceptors (Payne et al., 1990), neuronal cell line N1E-115 (Chueh and Gill, 1986), AR42J cells (Zhao and Muallem, 1990), and *Xenopus* oocytes (Parker and Ivorra, 1990*a*).

Of the experiments that demonstrate Ca^{2+} inhibition of Ca^{2+} release those of Parker and Ivorra, (1990*a*) are the most quantitative. Parker and Ivorra, (1990*a*) loaded unstimulated *Xenopus* oocytes with caged IP_3, activated a fraction of the caged IP_3 using short (0.01–0.1 s) pulses from an arc lamp, and recorded the change of $[Ca_i^{2+}]$, Δ. Using this technique, they also developed a two-pulse protocol in which two identical light pulses were used to initiate Ca^{2+} release, with the time interval between the pulses being varied. The ratio, Δ_2/Δ_1, was used to measure the effect that the first release of Ca^{2+} had on the second, with $\Delta_2/\Delta_1 < 1$ indicating inhibition. Plots of

Δ_2/Δ_1 versus the interpulse time interval were used to quantify the time course of the inhibition.

Using a Ca^{2+}-sensitive fluorescence indicator (fluo-3), they found that maximal inhibition occurred at ~2 s following the first pulse of IP_3 and that recovery from the first pulse was complete after 14–20 s. A significant amount of inhibition ($\Delta_2/\Delta_1 \approx 0.2$–$0.3$) was observed at maximum inhibition. Parker and Ivorra's work shows at least three time scales operating in *Xenopus* oocytes: a rapid (0.2–0.5 s) release of Ca^{2+} from an IP_3 sensitive pool; a slower (2–3 s) inhibition of Ca^{2+} release by Ca^{2+}; and a longer recovery process (14–20 s).

One possible explanation of their data is that the Ca^{2+} in the IP_3-sensitive store is exhausted during the first pulse and, hence, at the time of the second pulse the store has not completely refilled. Only when the store is completely refilled would the Ca^{2+} released by the second pulse approximate that in the first pulse. To eliminate this possibility Parker and Ivorra, (1990*a*) used pulses of IP_3 that were significantly smaller than those that produced maximal calcium release. In addition, they observed inhibition of the IP_3-induced Ca^{2+} release when Ca^{2+} was directly microinjected into the oocyte prior to IP_3 simulation (Parker and Ivorra, 1990*a*).

We propose here a simple model to account for Ca^{2+} inhibition that incorporates three important facts regarding the IP_3-receptor/Ca^{2+}-channel. First, the effective K_d for IP_3 binding to microsomal ER fractions is increased by Ca^{2+} (Joseph et al., 1989), from ~145 nM in the absence of calcium to 542 nM in the presence of 1 μM Ca^{2+}. Although there is evidence that suggests that the binding of IP_3 is regulated through a Ca^{2+}-binding protein (Supattapone et al., 1988), we assume for simplicity that Ca^{2+} directly affects the binding of IP_3. Second, the binding of IP_3 to its receptor is highly cooperative with a Hill coefficient of at least three (Meyer et al., 1988) and probably four (Meyer et al., 1990). Finally, we explicitly include the inhibitory effect of Ca^{2+} on Ca^{2+} release by the IP_3-receptor/Ca^{2+}-channel.

Ferris et al., 1989, suggest that the IP_3-receptor/Ca^{2+}-channel is a tetramer of four identical subunits. Kinetically, we model the IP_3-receptor as consisting of four independent and equivalent subunits (Meyer et al., 1990). Each subunit is endowed with an IP_3 and a Ca^{2+} binding site that interact with each other, such that when the Ca^{2+} site is occupied the K_d for binding of IP_3 is increased. Thus a subunit can exist in four states (see Fig. 1): state s_0 consists of a subunit with neither IP_3 nor Ca^{2+} bound; s_1 has only IP_3 bound; s_2 has both IP_3 and Ca^{2+} bound; and s_3 has only Ca^{2+} bound. An open channel is assumed to result only when each one of the four subunits is in the state s_1. All other states of the

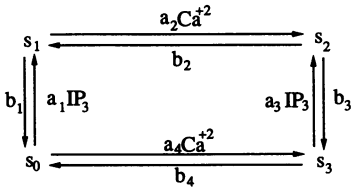


FIGURE 1 A kinetic diagram of the proposed states of an isolated subunit of the IP_3-receptor/Ca^{2+} channel.

tetramer are assumed to be closed. Thus a rise in $[Ca_i^{2+}]$ shifts the channel into a blocked state. Another way to incorporate the inhibitory effect of Ca^{2+} would be to assume that the tetramer remains open even with subunits in the s_2 state but that the rate of Ca^{2+} flux is reduced. For simplicity this possibility is ignored.

Assuming mass action kinetics the kinetic equations governing the state of a subunit are

$$\frac{dx_0}{dt} = -([IP_3]a_1x_0 - b_1x_1) - ([Ca_i^{2+}]a_4x_0 - b_4x_3)$$

$$\frac{dx_1}{dt} = ([IP_3]a_1x_0 - b_1x_1) - ([Ca_i^{2+}]a_2x_1 - b_2x_2)$$

$$\frac{dx_2}{dt} = ([Ca_i^{2+}]a_2x_1 - b_2x_2) + ([IP_3]a_3x_3 - b_3x_2)$$

$$\frac{dx_3}{dt} = ([Ca_i^{2+}]a_4x_0 - b_4x_3) - ([IP_3]a_3x_3 - b_3x_2), \qquad (1)$$

where x_i denotes the fraction of subunits in state s_i. The equilibrium state of these equations can be fit to the IP_3 binding data of Joseph et al., 1989. Defining $d_i = b_i/a_i$, we find that the K_d's with and without 1 μM Ca^{2+} satisfy the equations:

$$d_1 = K_{d1} - \overline{IP_3}$$
$$d_3 = (K_{d2} - \overline{IP_3})(1 + d_2) - d_1d_2, \qquad (2)$$

where $K_{d1} = 145$ nM is the effective K_d for IP_3 binding in the absence of Ca^{2+}, $K_{d2} = 542$ nM is the effective K_d for IP_3 binding in the presence of 1 μM Ca^{2+}, and $\overline{IP_3} = 15$ nM is the concentration of labeled IP_3 used in the cold titration process at 0°C (Joseph et al., 1989). In this notation, the thermodynamic constraint on the rate parameters (Hill, 1977) is $d_4 = d_1d_2/d_3$.

The flux of Ca^{2+} through the IP_3-mediated channel is proportional to the number of open channels and $[Ca_i^{2+}] - [Ca_{ER}^{2+}]$, where $[Ca_{ER}^{2+}]$ is the concentration of Ca^{2+} in the ER. Thus, the flux of Ca^{2+} through the IP_3 mediated channel is given by

$$v_1x_1^4([Ca_i^{2+}] - [Ca_{ER}^{2+}]),$$

where v_1 is the maximum flow rate through an open channel multiplied by the density of channels on the

surface of the ER. Other fluxes that we assume to be present are an outward leak, proportional to $[Ca_i^{2+}] - [Ca_{ER}^{2+}]$, and an inward flux that results from an ATP-dependent Ca^{2+} pump, proportional to $[Ca_i^{2+}]^2/([Ca_i^{2+}]^2 + k_4^2)$ (Carafoli, 1987). With these assumptions the kinetic equation governing $[Ca_i^{2+}]$ is

$$\frac{d[Ca_i^{2+}]}{dt} = c_2(v_1 x_1^4 + v_8)([Ca_{ER}^{2+}]] - [Ca_i^{2+}]) - v_4 \frac{[Ca_i^{2+}]^2}{[Ca_i^{2+}]^2 + k_4^2}, \quad (3)$$

where $c_2 = V_{ER}/V_{cyt}$ is the ratio of the ER volume to the volume of the cytosol. Using typical cell parameters (Alberts et al., 1989, 407–408) c_2 is set to 0.185. With the above model for the IP_3 receptor and its Ca^{2+} flux, the experiments by Parker and Ivorra can be modeled with the addition of a simple input and decay equation for IP_3,

$$\frac{d[IP_3]}{dt} = v_3 f(t) - v_7[IP_3], \quad (4)$$

where $f(t)$ takes on values of 0 or 1 and is used to define the pulses of IP_3 (the standard pulse width for $f(t)$ is c_9 = 0.05 ms). $[Ca_{ER}^{2+}]$ is determined by the Ca^{2+} conservation condition $c_0 = c_2[Ca_{ER}^{2+}] + [Ca_i^{2+}]$.

Due to a lack of quantitative measurements we have been unable to fix the parameters in this model by using experimental data from a single cell type. This has forced us to estimate parameter values using a variety of measurements made on several different cell types. Thus for the effect of Ca^{2+} on the binding of IP_3 to its ER receptor we have used the data of Joseph et al., 1989, on cerebellum microsomal fractions, as described below Eq. 2. For v_7, the decay rate of IP_3, we have used values of 1–3 s^{-1}, close to the value reported for rat basophilic leukemia (RBL) cells (Meyer and Stryer, 1988). Values of other parameters, such as those for the Ca^{2+}-ATPase (v_4 and k_4), the total free Ca^{2+} concentration (c_0), and the leak rate (v_8) have been chosen to insure that the resting value of cytosolic calcium agrees with typical experiments in a variety of cells (50–100 nM). The standard values that we have chosen are listed in Table 1 of the Appendix. Finally, the tetrameric structure for the ER IP_3 receptor and the order of magnitude of transition rates are based on kinetic experiments with permeabilized RBL cells (Meyer et al., 1990) and reconstitution experiments of Ferris, et al., 1989. As a consequence, it is not possible to argue that our calculations provide a model of a specific cell type. Nonetheless, we believe that the parameters that we use are of the correct order of magnitude and that calculations with our model may provide some useful, general insights.

The remaining parameters of the model are those describing the kinetics of the IP_3 receptor. We have selected the values of those parameters to reproduce the one- and two-pulse protocols of Parker and Ivorra, 1990*a*. As shown in Fig. 2, it is possible to do this rather well using the standard parameters listed in Table 1 (Appendix). In agreement with the experiment, a single pulse of IP_3 (*dashed line*) yields a rapid rise in $[Ca_i^{2+}]$ to a maximum value in ~0.5 s. This is followed by a slower decrease to the original resting level. The inhibition caused by a subsequent pulse, as measured by Δ_2/Δ_1, is shown as the solid line. In agreement with experiment the maximum inhibition in the two-pulse protocol occurs at $\Delta_2/\Delta_1 \approx 0.25$ and ~2 s after the first pulse of IP_3. Recovery is essentially complete ($\Delta_2/\Delta_1 \approx 0.95$) after 20 s (Fig. 2*A*). Furthermore, the size of the Ca^{2+} pulse evoked by a single pulse of IP_3 depends nonlinearly on the duration of the IP_3 input pulse. This is shown in Fig. 2 *B* where a threshold pulse duration of ~20 ms is required to elicit a significant pulse of Ca^{2+}. This is similar to the behavior seen by Parker and Ivorra, 1990 *b*, although the threshold is not as sharp as seen experimentally. For two IP_3 pulses of short duration we are also able to reproduce the facilitation effect (i.e., $\Delta_2/\Delta_1 > 1$) seen by Parker and Ivorra, 1990*a*. In our model, facilitation is due to the fact that small releases of Ca^{2+} are insufficient to cause appreciable inhibition.

We should note here that the standard parameters in Table 1 are not unique, in the sense that certain groups or pairs of parameters can be changed in concert to give results similar to those in Fig. 2. However, in the absence of comprehensive measurements for a single cell type, we have not attempted to optimize the parameter values, but rather take the standard values in Table 1 as a standard reference.

It is easy to describe the sequence of events involved in a single Ca^{2+} spike: Before a pulse of IP_3 is given, the receptor subunits are predominately in state s_0 or s_3, depending on the basal level of $[Ca_i^{2+}]$, with those channels that have all subunits in state s_0 being poised to open. Once the IP_3 pulse has been discharged, the subunits in state s_0 begin to shift to state s_1; a Ca^{2+}-channel opens when all four subunits are in the s_1 state; and the outward flow of calcium begins. As $[Ca_i^{2+}]$ increases, the subunits switch to state s_2, thereby blocking open channels. While the channels are blocked, IP_3 degrades and the ATP-dependent Ca^{2+} pump recycles the Ca^{2+} back into the ER. Inhibition is, therefore, a direct result of the increase in $[Ca_i^{2+}]$. The delay of maximum inhibition results from Ca^{2+} binding to state s_1 and the slow transition to state s_2. Recovery is complete when $[Ca_i^{2+}]$ is returned to its basal level by the pump and leak current and sufficient time has elapsed to

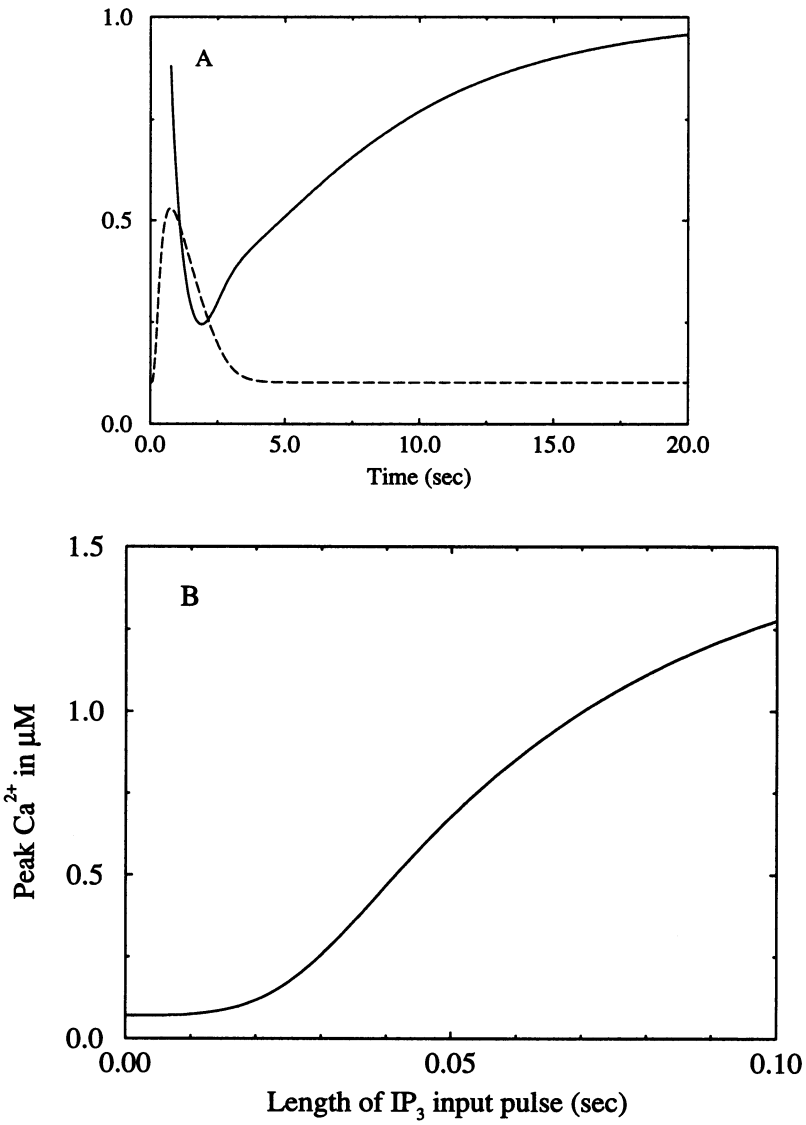


FIGURE 2 The results of a simulation using Eqs. 3 and 4. (*A*) Solid line, Δ_2/Δ_1 vs interpulse interval; dashed line, the Ca^{2+} transient resulting from a single pulse of IP_3. (*B*) The IP_3-Ca^{2+} dose response curve given in terms of the length of the IP_3 input pulse and maximum peak $[Ca_i^{2+}]$. Parameters: standard parameters with $v_6 = 0.0$ and $v_7 = 1.0$.

repopulate the s_0 and s_3 states. Not surprisingly, we have found that the degree of inhibition is directly related to the peak $[Ca_i^{2+}]$ and the width of the Ca^{2+} transient. Thus, the parameters that control Ca^{2+} efflux and influx into the ER along with the rate of IP_3 degradation have the greatest effects on the degree and timing of the inhibition.

Due to the large dimension of the parameter space for these equations (10), it is difficult to state categorically that Eqs. 1 and 3 do not admit periodic solutions. However, our searches of parameter space have turned up no set of values for which the steady state is unstable. Intuitively, one might expect this, because there is nothing in these equations to counter the inhibitory mechanism, which can only dampen the release of calcium, thereby maintaining homeostasis. We conjecture that any mechanism involving Ca^{2+} inhibition of Ca^{2+} release must also include positive feedback on the release of Ca^{2+} to produce Ca^{2+} oscillations. The positive feedback may take the form of Ca^{2+} acting as a coagonist of the IP_3 receptor (Bezprozvanny et al., 1991, Finch et al., 1991) or an indirect effect on the IP_3 production. In the remainder of this paper we explore the second possibility.

FEEDBACK OF Ca^{2+} ON IP_3 PRODUCTION

There is an on going debate about whether the IP_3 concentration within a cell is constant (Wakui et al., 1989) or varies in phase with the Ca^{2+} oscillations (Harootunian et al., 1991). It is clear, however, that the production of IP_3 can be stimulated by $[Ca_i^{2+}]$. Indeed, the stimulation of IP_3 production by Ca^{2+}, if sufficiently rapid, may even account for some observations of Ca^{2+}-induced Ca^{2+} release (Mouillac et al., 1990).

The production of IP_3 occurs through phosphatidylinositol 4,5-bisphosphate-PLC (PIP_2-PLC) activity (Taylor and Exton, 1987). PLC is activated by a specific α_q subunit of a G-protein, G_q (Smrcka et al., 1991). Recent experiments (Mouillac et al., 1990, Smrcka et al., 1991, Taylor and Exton, 1987) indicate that intracellular Ca^{2+} modulates the production of IP_3; however, it is not clear whether this is a result of Ca^{2+} affecting PLC (Mouillac et al., 1990) or the α_q subunit (Smrcka et al., 1991). In either case, the level of $[Ca_i^{2+}]$ that produces a half maximal IP_3 production lies within physiological ranges 0.1–3.0 μM. This suggests the possibility of a positive feedback mechanism of $[Ca_i^{2+}]$ on the production of IP_3 similar to that invoked by Meyer and Stryer, 1988.

Thus, we extend Eq. 4 to include a simple hyperbolic stimulation of IP_3 by Ca^{2+}. Eq. 4 now becomes

$$\frac{d[IP_3]}{dt} = v_3 f(t) + v_6 \frac{[Ca_i^{2+}]}{k_6 + [Ca_i^{2+}]} - v_7[IP_3], \qquad (5)$$

where v_6 represents the maximum production rate of IP_3. The constant v_6 incorporates effects on the production of IP_3 that are independent of $[Ca_i^{2+}]$, such as, the concentration of PIP_2 in the plasma membrane, the concentration of α_q, et cetera. The parameter v_6 serves, therefore, to indicate the level of agonist stimulation of receptors in the plasma membrane. Fig. 3 shows a schematic of the calcium fluxes and the feedback loops of our model.

By combining the receptor dynamics in Eq. 1 with Eqs. 3 and 5, it is possible to reproduce the inhibition experiments and produce oscillations in $[Ca_i^{2+}]$ without modifying the receptor kinetics. Results similar to Fig. 2 can be attained by assuming a basal level of IP_3 production, $v_6 = 0.22$ μM s^{-1}, and doubling the IP_3 decay rate, $v_7 = 2.0$ s^{-1}. As agonist stimulation is increased by elevating v_6, the steady state that characterizes the basal Ca^{2+} level becomes unstable and periodic solutions arise from a Hopf bifurcation. Analysis with AUTO (Doedel and Kernevez, 1986), shows that periodic solutions exist for $v_6 = 0.624$ to 0.710 with periods varying from ~19–15.5 s, as shown in Fig. 4*A*. Fig. 4*B* shows the periodic solution for $v_6 = 0.69$.

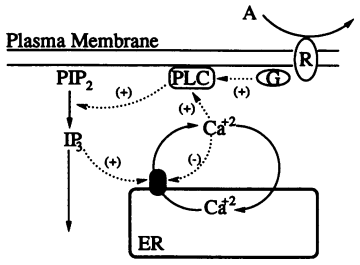


FIGURE 3 A schematic of the proposed model. The solid lines represent fluxes and the dashed lines represent feedback mechanisms. The calcium efflux from the ER is hypothesized to be proportional to $x_1^4([Ca_i^{2+}] - [Ca_{ER}^{2+}])$, where x_1 is the fraction of subunits in state s_1. The influx is the result of a ATP-dependent Ca^{2+}-pump and is hypothesized to be proportional to $[Ca_i^{2+}]^2/([Ca_i^{2+}]^2 + k_2^2)$. Also assumed, but not shown, is a leak Ca^{2+} efflux. IP_3 is produced by the action of PLC on PIP_2.

ANALYSIS AND SIMPLIFICATION OF THE MODEL

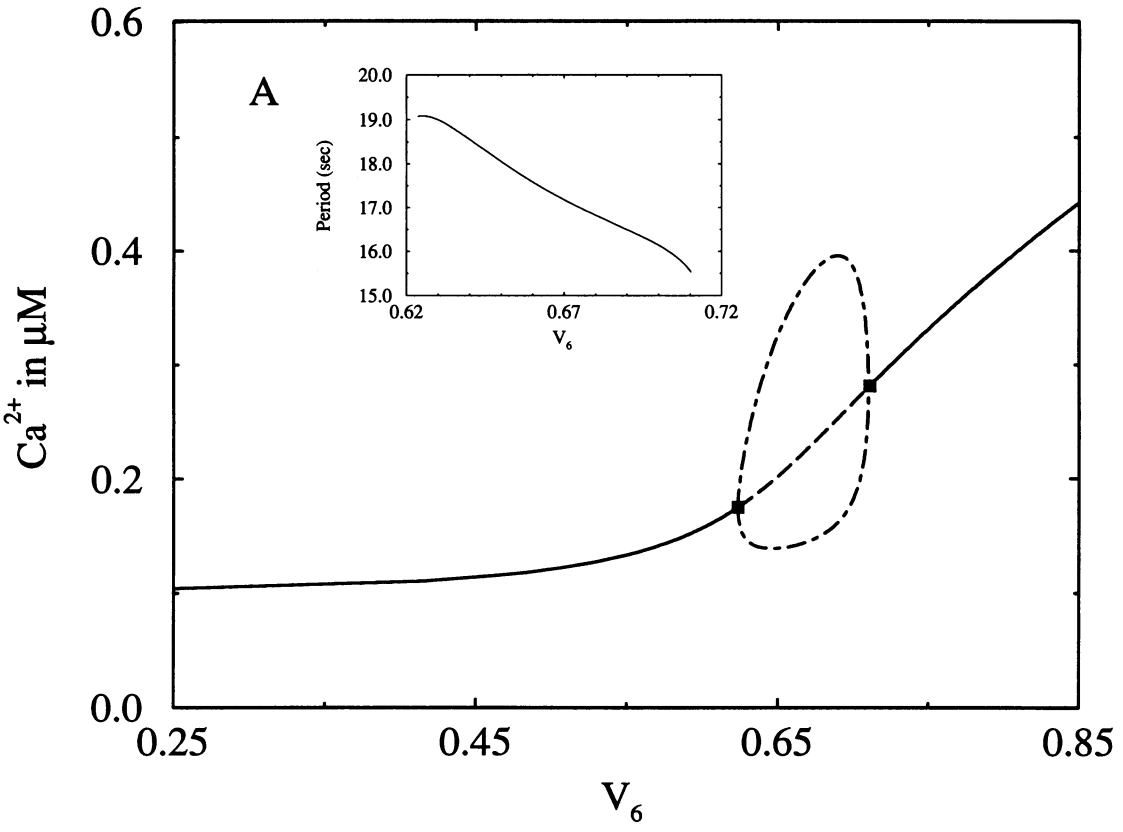
In an effort to understand which components of the model are the most important, we have investigated several simplifications of it. The rationale for the simplifications is based on an analysis of time scales in the full model. In a dynamical system, $dy/dt = F(y)$, where y and $F(y)$ are vectors, the instantaneous rate of relaxation of y_i at time t can be defined as

$$V_{y_i}(t) = \frac{F_i(y(t))}{y_i^{\infty}(t) - y_i(t)},$$

where $y_i^{\infty}(t)$ is the solution to $0 = F_i(y)$ with $y_k = y_k(t), k \neq i$, fixed. A simple algebraic computation shows that

$$\begin{aligned} V_{IP_3} &= v_7, \\ V_{x_0}(t) &= [IP_3]a_1 + [Ca_i^{2+}]a_4, \\ V_{x_1}(t) &= [Ca_i^{2+}]a_2 + b_1, \\ V_{x_2} &= b_2 + b_3, \\ V_{x_3}(t) &= [IP_3]a_3 + [Ca_i^{2+}]b_4. \end{aligned} \qquad (6)$$

An expression for $V_{Ca^{2+}}$ is not easily found by algebraic manipulation. We have calculated the instantaneous rates of relaxation for each variable along a periodic solution for the standard parameters ($v_6 = 0.69$) and have found that V_{x_1} and V_{x_2} are consistently greater than the other time scales; thus x_1 and x_2 track their pseudo steady state values (y_i^{∞}) better than x_0, $[Ca_i^{2+}]$, and $[IP_3]$.



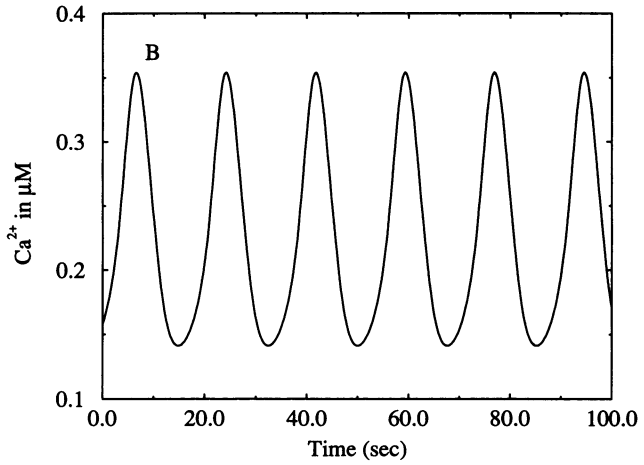


FIGURE 4 (*A*) A bifurcation diagram using Eqs. 1, 3, and 5 with v_6 as the bifurcation parameter. The solid and dashed lines give the stable and unstable steady states respectively as a function of v_6. The closed squares are Hopf bifurcation points. The dash-dotted line gives the maximum and minimum along the periodic orbits. The inset shows the period of the periodic solution as a function of v_6. (*B*) The periodic solution for $v_6 = 0.69$. Parameters: standard parameters.

Eq. 6 shows that $V_{x_1}(t) > b_1$ and $V_{x_2} > b_3$ for all t. This suggest taking the limits $b_1 \to \infty$ and $b_3 \to \infty$ while holding d_1 and d_3 fixed. In this limit x_1 and x_2 relax instantaneously to their quasi-steady state values

$$\bar{x_1} = \frac{[IP_3]x_0}{d_1}$$
$$\bar{x_2} = \frac{[IP_3]x_3}{d_3}. \tag{7}$$

Using the receptor conservation condition $x_3 = 1 - x_0 - x_1 - x_2$, gives

$$\bar{x_2} = \frac{[IP_3](1 - x_0 - \bar{x_1})}{[IP_3] + d_3}. \tag{8}$$

In this limit, our model simplifies considerably. Using Eqs. 7 and 8 the simplified model is seen to involve only three dynamical variables, namely, x_0, Ca^{2+}, and IP_3. With this reduction our simplified three-variable model

takes the form

$$\frac{d[Ca_i^{2+}]}{dt} = c_2(v_1(\bar{x}_1)^4 + v_8)([Ca_{ER}^{2+}] - [Ca_i^{2+}]) - v_4\frac{[Ca_i^{2+}]^2}{[Ca_i^{2+}]^2 + k_4^2},$$

$$\frac{d[IP_3]}{dt} = v_6\frac{[Ca_i^{2+}]}{k_6 + [Ca_i^{2+}]} - v_7[IP_3],$$

$$\frac{dx_0}{dt} = -a_4[Ca_i^{2+}]x_0 + b_4(1 - x_0 - \bar{x}_1 - \bar{x}_2) \quad (9)$$

where $[Ca_{ER}^{2+}] = (c_0 - [Ca_i^{2+}])/c_2$. The system (Eq. 9) may be thought of in terms of two opposing roles for Ca^{2+}, both represented in $\bar{x}_1 = [IP_3]x_0/d_1$. The first role is a delayed inhibition through the dynamics of the unoccupied receptor, x_0. The second role is that of indirect Ca^{2+}-induced Ca^{2+} release through the stimulation of IP_3 production.

The role of Ca^{2+} in indirectly inducing Ca^{2+} release may be seen more clearly if we assume that IP_3 also varies on a rapid time scale. Bifurcation analysis with AUTO using $\alpha = v_6/v_7$ and $V_{IP_3} = v_7$ as parameters shows that periodic solutions of system (Eq. 9) arising from Hopf bifurcations cease to exist only when V_{IP_3} is less than 0.5742. That is, the feedback induced release of Ca^{2+} must occur on a faster time scale than the inhibition for oscillations to exists. By letting $V_{IP_3} \to \infty$ while holding α fixed, $[IP_3]$ instantaneously tends to its quasi-steady state,

$$[\overline{IP_3}] = \alpha\left(\frac{[Ca_i^{2+}]}{[Ca_i^{2+}] + k_6}\right).$$

Setting $[IP_3]$ equal to $[\overline{IP_3}]$, produces the final simplification, i.e., a two-variable model in which the outward flux of calcium through the IP_3-mediated channel in system (Eq. 9) is given by

$$x_0^4 v_m\left(\frac{[Ca_i^{2+}]}{[Ca_i^{2+}] + k_6}\right)^4([Ca_{ER}^{2+}] - [Ca_i^{2+}]), \quad (10)$$

where $v_m = c_2v_1\alpha^4/d_1^4$. It should be noted that (Eq. 10) is similar to the outward Ca^{2+} flux term used by Dupont and Goldbeter, 1989, in their two pool model, i.e.,

$$V_{M3}\left(\frac{[Ca_i^{2+}]^4}{[Ca_i^{2+}]^4 + k_R^4}\right)\left(\frac{[Ca_{ER}^{2+}]^2}{[Ca_{ER}^{2+}]^2 + k_A^2}\right). \quad (11)$$

The two-variable simplification of the full model may be thought of as a pseudo Ca^{2+}-induced Ca^{2+} release model modified by inhibition. The major differences with the direct models of Ca^{2+}-induced Ca^{2+} release (Dupont and Goldbeter, 1989, Dupont et al., 1991) being the bidirectionality of the outward Ca^{2+} flux and the Ca^{2+} feedback on the production of IP_3.

The pseudo Ca^{2+}-induced Ca^{2+} release found in the simplified models reflects the indirect effect of Ca^{2+} on its own release via stimulation of IP_3 production. This can be shown explicitly by calculating the response of Ca^{2+} to a single square-wave pulse of Ca^{2+} that is applied at steady state. In Fig. 5 we show the results of such calculations with the three-variable model in which the strength of the agonist stimulation, v_6, was set somewhat below the value that produces oscillations. Notice that Ca^{2+} pulses of short duration decay within a few seconds back to their steady state value. Beyond a certain critical value of the pulse length, however, a distinct secondary pulse of Ca^{2+} is found. While this has the appearance of Ca^{2+}-induced Ca^{2+} release, it is only apparently so. Indeed, the real cause can be traced back to the slow kinetics of state s_0 of the IP_3 receptor.

This is shown more clearly in the inset to Fig. 5, where the nullclines in the two-variable model, obtained by setting $d[Ca_i^{2+}]/dt = 0$ and $dx_0/dt = 0$, are shown as the solid lines. The intersection of the two nullclines defines the steady state, which is stable for the parameter values shown. By increasing α the intersection moves between the two knees of the N-shaped Ca^{2+} nullcline into an unstable region and oscillations occur. However, when the intersection is close to the left knee, as shown in the inset, a pulse of Ca^{2+} of sufficient size can force $[Ca_i^{2+}]$ into the region well beyond the middle branch of the Ca^{2+} nullcline. In this region the Ca^{2+} concentration increases without much change in x_0, producing the secondary Ca^{2+} pulse, as indicated by the long dashed trajectory. Smaller initial pulses may be insufficient to produce a significant secondary pulse (short dashed

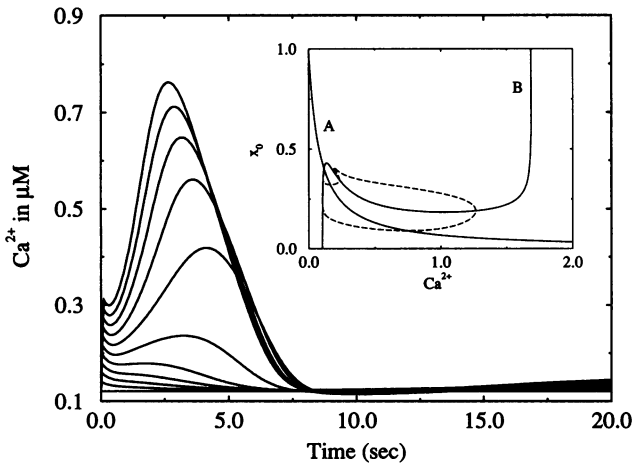


FIGURE 5 Responses of the three-variable model to a Ca^{2+} pulse of increasing length (0.0–0.1 s). The inset shows the phase portrait in the limit $V_{IP_3} \to \infty$, dashed lines: two trajectories resulting from different calcium pulses, solid lines: the nullclines $dx_0/dt = 0$ (A) and $d[Ca_i^{2+}]/dt = 0$ (B). Parameters: standard parameters with $\alpha = 0.23$ and $v_7 = 2.0$.

trajectory) or, if they fall short of the middle branch, no secondary pulse at all. This type of threshold behavior is like that seen for the stimulation of action potential spikes and has the same mathematical origin (Rinzel and Ermentrout, 1991).

It is easy to show that Ca^{2+} oscillations exist in the two-variable model (using the standard parameters with α variable); however, because the feedback of Ca^{2+} on Ca^{2+} release is instantaneous, the outward flux of Ca^{2+} is much larger and ER Ca^{2+} is typically exhausted near the peak of the Ca^{2+} oscillation. The ER remains in an exhausted state until the inhibition (x_0) has sufficient time to respond to the increased level of cytosolic Ca^{2+}, at which point the Ca^{2+} channel becomes blocked and outward flux of Ca^{2+} is then reduced. On the other hand, a reduced two-variable model in which x_0, x_1, and x_2 are all set equal to their quasi-steady-state values, leaving only Ca^{2+} and IP_3 as variables, does not support oscillations. This makes it clear that the timing of the dynamics of the IP_3 receptor complex, chiefly through the state s_0, is responsible for producing oscillations in our model.

For the three-variable model, Eq. 9, inhibition plays an active role in limiting the size of the Ca^{2+} transient when $v_7 < 2\ s^{-1}$. Because the receptor kinetics of IP_3 are assumed instantaneous, lower levels of IP_3 are required for an equivalent Ca^{2+} transient of the full model. Fig. 6 *A* shows the two pulse experiment for the system (Eq. 9) with $v_6 = 0.11\ \mu M^{-1}\ s^{-1}$, $v_7 = 1\ s^{-1}$, and $c_9 = 0.025$ s. For the three-variable model Fig. 6 *B* shows a representative periodic solution for $v_6 = 0.34$. Fig. 7 shows the envelope of periodic solution and their periods (*inset*) as a function of v_6 for $v_7 = 1\ s^{-1}$.

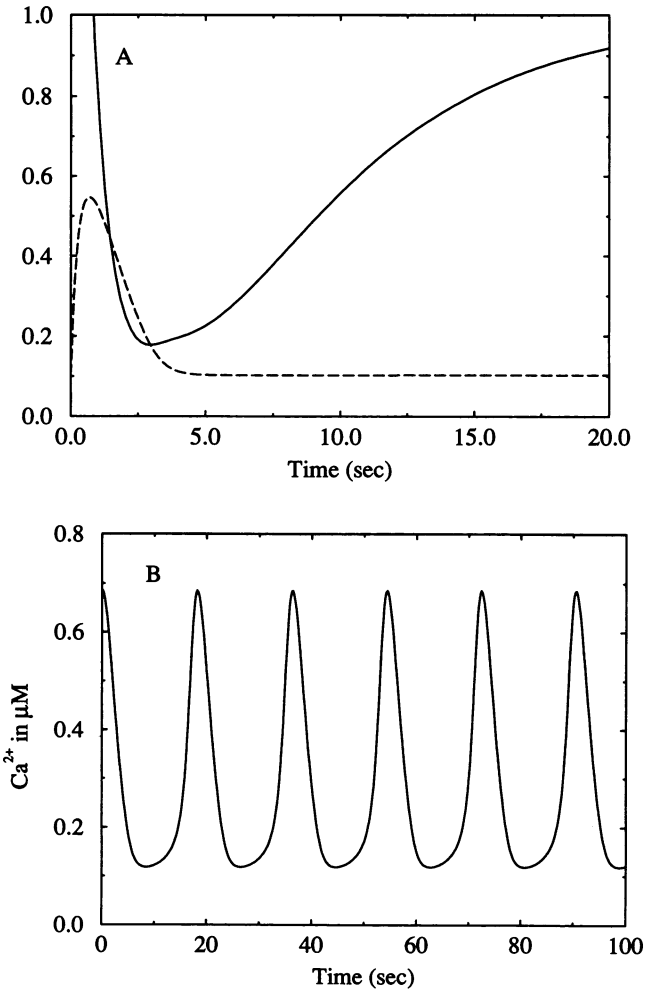


FIGURE 6 (*A*) The two pulse protocol of Parker and Ivorra, 1990*a*, using system (9), (*solid line*) Δ_2/Δ_1 vs interpulse interval, (*dashed line*) the Ca^{2+} transient resulting from a single pulse of IP_3, $v_6 = 0.11$, $c_9 = 0.025$. (*B*) The periodic solution of system (9) for $v_6 = 0.34$. Parameters: standard parameters with $v_7 = 1.0$.

DISCUSSION

Our main point in this work has been to show that Ca^{2+} inhibition of Ca^{2+} release from the endoplasmic reticulum may play an active role in agonist-stimulated Ca^{2+} oscillations. In our mechanism the binding of IP_3 to receptors on the ER membrane opens Ca^{2+} channels, which in turn release calcium into the cytoplasm. In the presence of agonist-stimulated G_q protein, our mechanism invokes two roles for Ca^{2+}: (*a*) inhibition of Ca^{2+} release from the ER and (*b*) stimulation of the production of IP_3 through the action of PLC on PIP_2. These two processes affect Ca^{2+} release in opposing ways, the first inhibiting it and the second stimulating it. Over a range of values for agonist-stimulation, we find that these opposing effects lead to limit cycle type oscillations in cytosolic Ca^{2+}.

Our analysis of the kinetic equations that describe this mechanism shows that oscillations exist only for an intermediate range of agonist stimulation, in agreement with experiments on hepatocytes (Woods et al., 1986). We also find that Ca^{2+} and IP_3 oscillate in phase, as has been suggested to be the case in REF52 cells by synchronization experiments (Harootunian et al., 1991). Another interesting observation that comes from our calculations is that in the presence of agonist concentrations slightly below those that produce oscillations, a short pulse of Ca^{2+} can invoke a much larger secondary pulse. Using caged-Ca^{2+} this sort of phenomenon has been observed recently in REF52 fibroblasts (Harootunian et al., 1991). Interestingly, those cells failed to exhibit a secondary pulse of Ca^{2+} either in the absence of agonist or in the presence of the IP_3-receptor blocker, heparin. Our model provides a simple explanation of these observations, namely, in the presence of agonist

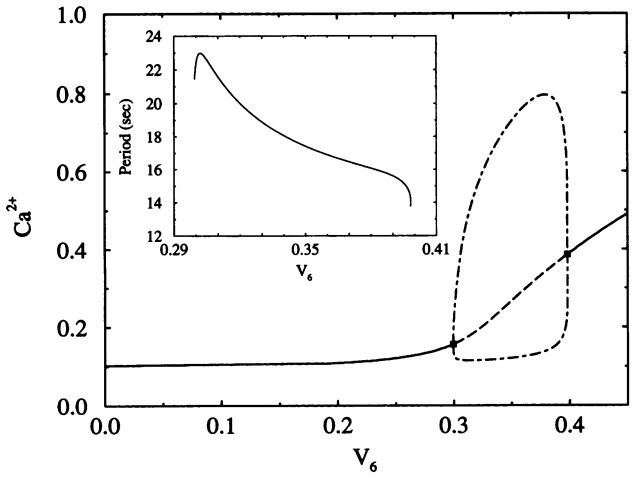


FIGURE 7 Bifurcation diagram for system (9) with v_6 as the bifurcation parameter. The solid and dashed lines give the steady states with the dashed line representing unstable steady states. The closed squares are Hopf bifurcation points. The dash-dotted line gives the maximum and minimum along the periodic orbits. (*Inset*) The period of the periodic solution as a function of v_6. Parameters: standard parameters with $v_7 = 1.0$.

the initial Ca^{2+} pulse stimulates the PLC-catalyzed breakdown of PIP_2 into IP_3, which in the absence of heparin releases a secondary pulse Ca^{2+} from the ER. This indirect form of Ca^{2+}-induced Ca^{2+}-release, illustrated in Fig. 5, is quite different from the direct form that has been postulated to exist in a number of cell types (Dupont and Goldbeter, 1989, Berridge, 1991). We find this pseudo Ca^{2+}-induced Ca^{2+}-release to be more pronounced when the removal rate of IP_3 is increased and wonder along with Mouillac et al., 1990, if in some cell types it might not have been mistaken inadvertently for the direct process.

Changes in several of the kinetic parameters in our model lead to significant changes in the oscillations. For example, if the dissociation constant for Ca^{2+} binding to the Ca^{2+}-ATPase, k_4, is decreased, the amplitude and period of the oscillations increases until a critical point is reached at which the oscillations disappear. The existence of oscillations is also quite sensitive to the maximal rate of the Ca^{2+}-ATPase, v_4, and if this parameter is decreased the oscillations also are lost. In light of this it might be interesting to carry out experiments using trace amounts of thapsigargin to partially inhibit the Ca^{2+}-ATPase in the ER (Thastrup et al., 1990). Our calculations predict that this would eliminate agonist-stimulated oscillations occurring by the mechanism described above. The oscillations are also sensitive to the rate of leak of Ca^{2+} from the ER (v_8 in our model) and would appear to be eliminated by most changes that increase net efflux of Ca^{2+} from the ER into the cytosol.

The model also makes quantitative predictions about the effect of agonist or of defined pulses of Ca^{2+} on the release of Ca^{2+} by pulses IP_3. Increasing the level of stimulation of agonist close to the threshold level for oscillations, we find that the amount of inhibition in the two-pulse protocol of Parker and Ivorra, 1990*a*, (cf Fig. 2*A*) can be increased so that a second peak of Ca^{2+} no longer can be seen. This is a consequence of the additional IP_3 that is produced via the PLC step during the first pulse when sufficient agonist is present. It is also possible to simulate mixed pulse protocols with our model, e.g., an initial pulse of Ca^{2+} (from caged-Ca^{2+}) followed by a pulse of IP_3 (from caged-IP_3). These sort of experiments should be easy to perform in the laboratory and could help confirm or refute the basic kinetic features of the model.

TABLE 1 The standard parameter set

Parameter	Value	Unit	Description
v_1	800	s^{-1}	Ca^{2+} channel flux constant
v_3	2.0	$\mu M\ s^{-1}$	External IP_3 input rate
v_4	0.5	$\mu M\ s^{-1}$	Maximum Ca^{2+} uptake rate
v_6	Variable	$\mu M\ s^{-1}$	Maximum Ca^{2+} dependent IP_3 input rate
v_7	2.0	s^{-1}	IP_3 decay rate constant
v_8	0.15	s^{-1}	Ca^{2+} leak flux constant
k_4	0.09	μM	Activation const. ATP-dependent Ca^{2+} pump
k_6	1.1	μM	Activation const. Ca^{2+} dependent IP_3 input
a_1	50	$\mu M^{-1}\ s^{-1}$	Receptor parameter
a_2	1.0	$\mu M^{-1}\ s^{-1}$	Receptor parameter
a_3	20.0	$\mu M^{-1}\ s^{-1}$	Receptor parameter
a_4	0.9	$\mu M^{-1}\ s^{-1}$	Receptor parameter
b_1	6.5	s^{-1}	Receptor parameter ($= d_1a_1$)
b_2	0.5	s^{-1}	Receptor parameter
b_3	14.5	s^{-1}	Receptor parameter ($= d_3a_3$)
b_4	0.0806	s^{-1}	Receptor parameter ($= d_4a_4$)
c_0	2.0	μM	Total Ca^{2+} in terms of cytosolic volume
c_2	0.185	—	(Volume of ER)/(Volume of cytosol)
c_9	0.05	s	External IP_3 pulse length
K_{d1}	0.145	μM	Effective K_d for IP_3 binding with $[Ca_i^{2+}] = 0\mu M$
K_{d2}	0.542	μM	Effective K_d for IP_3 binding with $[Ca_i^{2+}] = 1\mu M$
$\overline{IP_3}$	0.015	μM	

Parameters b_1, b_3, and b_4 are determined by constraints given in the text and are listed only for completeness.

APPENDIX

Parameters

A complete list of the parameters used in the simulations, unless otherwise noted, is given in Table 1.

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