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Molecular model for receptor-stimulated calcium spiking

(inositol phospholipid cascade/inositol trisphosphate/calcium channels/oscillations/frequency encoding)

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ABSTRACT **Many cells exhibit periodic transient increases in cytosolic calcium levels rather than a sustained rise when stimulated by a hormone or growth factor. We propose here a molecular model that accounts for periodic calcium spiking induced by a constant stimulus. Four elements give rise to repetitive calcium transients: cooperativity and positive feedback between a pair of reciprocally coupled (crosscoupled) messengers, followed by deactivation and then by reactivation. The crosscoupled messengers in our model are inositol 1,4,5-trisphosphate ($InsP_3$) and cytosolic calcium ions. The opening of calcium channels in the endoplasmic reticulum by the binding of multiple molecules of $InsP_3$ provides the required cooperativity. The stimulation of receptor-activated phospholipase C by released calcium ions leads to positive feedback. $InsP_3$ is destroyed by a phosphatase, and calcium ion is pumped back into the endoplasmic reticulum. These processes generate bistability: the cytosolic calcium concentration abruptly increases from a basal level to a stimulated level at a threshold degree of activation of phospholipase C. Spiking further requires slow deactivation and subsequent reactivation. In our model, mitochondrial sequestration of calcium ion prevents the cytosolic level from increasing above several micromolar and enables the system to return to the basal state. When the endoplasmic reticulum calcium store is refilled to a critical level by the Ca^{2+}-ATPase pump, cooperative positive feedback between the $InsP_3$-gated channel and phospholipase C begins again to give the next calcium spike. The time required for the calcium level in the endoplasmic reticulum to reach a threshold sets the interval between spikes. The amplitude, shape, and period of calcium spikes calculated for this model are like those observed experimentally.**

Many signal transduction pathways are mediated by increases in cytosolic calcium levels produced by the action of the inositol phospholipid cascade (1, 2). The receptor-triggered hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C (PLC) results in the formation of inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol. $InsP_3$ then releases calcium ion from the endoplasmic reticulum (ER) by opening calcium channels. Woods, Cuthbertson, and Cobbold (3, 4) made the striking finding that hormone-stimulated hepatocytes exhibit repetitive transient rises in the concentration of calcium in the cytosol. They monitored the calcium level of individual cells by measuring the luminescence of microinjected aequorin, a calcium indicator. The addition of vasopressin, a hormone known to activate the inositol phospholipid cascade, led to repetitive increases of cytosolic calcium from 200 nM to about 1 μM, each lasting about 7 s. The frequency of spiking increased when the concentration of hormone was raised; the interval between spikes decreased from 150 s to 20 s. In contrast, the peak calcium level and the duration of each spike stayed nearly constant. Similar periodic calcium transients occur in many cell types, such as oocytes, mast cells, pituitary cells, and *Physarum polycephalum* (5–8).

These intriguing findings raise two fundamental questions: (*i*) How does a constant stimulus lead to periodic calcium spiking? The challenge is to unravel the molecular mechanism by which the *amplitude* of a hormonal stimulus determines the *frequency* of an intracellular response. (*ii*) What is the biological significance of calcium spiking? This article focuses on the first question and touches on the second.

Crosscoupled Messengers: $InsP_3$ and Ca^{2+}. The interacting messengers in our model are $InsP_3$ and cytosolic Ca^{2+} (Fig. 1). The steepness of the rising phase of a calcium spike suggests that a small increase in the concentration of $InsP_3$ resulting from activation of PLC leads to a large increase in the cytosolic level of calcium. Indeed, our recent studies of the kinetics of Ca^{2+} release from ER stores in permeabilized rat basophilic leukemia (RBL) cells (a tumor mast cell line) have shown that $InsP_3$ acts cooperatively (9). At least three molecules of $InsP_3$ must be bound to open a calcium channel. Cooperativity is necessary but not sufficient to give the steep rising phase. Calcium release must be reinforced by positive feedback. One possibility is that Ca^{2+} stimulates PLC, the enzyme that generates $InsP_3$ from phosphatidylinositol 4,5-bisphosphate (10, 11).

Both messengers are formed and removed. $InsP_3$ can be hydrolyzed to 1,4-inositol bisphosphate or phosphorylated to 1,3,4,5-inositol tetrakisphosphate (12). In permeabilized RBL cells containing 1 μM Ca^{2+} and 1 mM Mg^{2+}-ATP, we found that more $InsP_3$ is removed by hydrolysis than by phosphorylation. Cytosolic Ca^{2+} is pumped back into ER stores by a Ca^{2+}-ATPase. As will be discussed later, cytosolic Ca^{2+} is also taken up by mitochondria and pumped out of cells by transport systems in the plasma membrane.

The steps carried out by the simplified system shown in Fig. 1 can be expressed quantitatively. J_1, the $InsP_3$-induced flux of calcium out of the ER, is given by

$$J_1 = c_1 f z, \qquad [1]$$

where c_1 is the efflux rate constant, f is the fraction of channels open, and z is the concentration of Ca^{2+} in the ER (9). Our studies of $InsP_3$-induced release of Ca^{2+} in RBL cells (9) have shown that f can be approximated by

$$f = y^3/(K_1 + y)^3, \qquad [2]$$

where y is the $InsP_3$ concentration, and K_1 is the $InsP_3$ concentration at which half the sites are filled. K_1 is of the order of 100 nM and c_1 is 6.64 s^{-1}.

Calcium is pumped back into the ER by a Ca^{2+}-ATPase that is thought to transport two Ca^{2+} ions per reaction cycle (2). The flux J_2 into the ER is given by

$$J_2 = c_2 x^2/(x + K_2)^2 - c_3 z^2, \qquad [3]$$

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Abbreviations: ER, endoplasmic reticulum; $InsP_3$, inositol 1,4,5-trisphosphate; PLC, phospholipase C.

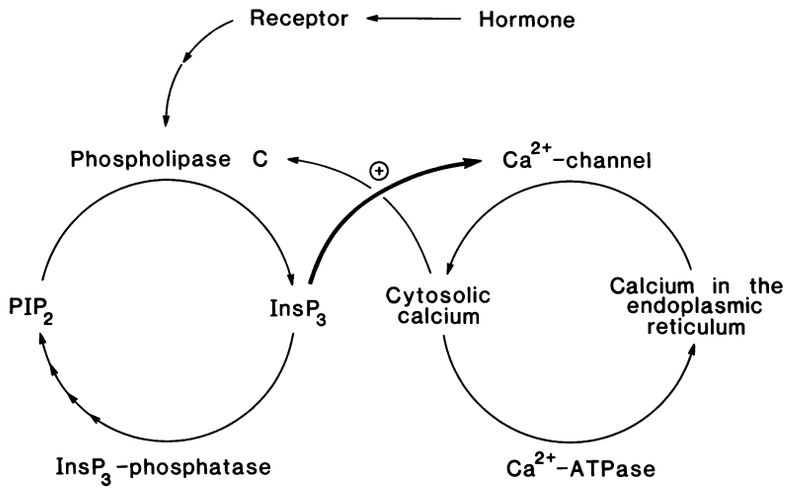

FIG. 1. $InsP_3$ and cytosolic Ca^{2+} are the two crosscoupled messengers. $InsP_3$ induces the release of Ca^{2+} from ER stores into the cytosol. The bold line emphasizes that $InsP_3$ cooperatively opens calcium channels in the ER. Cytosolic Ca^{2+}, in turn, stimulates the formation of $InsP_3$ from phosphatidylinositol 4,5-bisphosphate (PIP_2) by receptor-triggered PLC. Both messengers are cycled. $InsP_3$ is degraded by a phosphatase and subsequently converted to PIP_2. Cytosolic Ca^{2+} is pumped back into the ER by a Ca^{2+}-ATPase. Other processes controlling the cytosolic level of Ca^{2+} are not shown here.

where x is the cytosolic Ca^{2+} level and z is the ER Ca^{2+} level. The c_2 term is the ATP-driven pumping of Ca^{2+} into the ER, which is thought to be proportional to the square of the cytosolic Ca^{2+} concentration at low Ca^{2+} concentrations (13). The transport rate of the similar sarcoplasmic reticulum Ca^{2+}-ATPase is reported to be half-maximal at calcium concentrations ranging from about 0.1 to 1 μM (2). We use here a value of 0.36 μM, which corresponds to a K_2 value of 0.15 μM. The loading capacity of the ER is limited, probably by reversal of the ATPase at high ER Ca^{2+} levels. For simplicity, we take account of this reversal by a negative term that is proportional to the square of the ER Ca^{2+} level. By using a value of 3.13×10^{-5} $\mu M^{-1} \cdot s^{-1}$ for c_3, calculated ER Ca^{2+} levels are similar to those observed by us; at an external calcium concentration of 150 nM, $\approx$200 μM Ca^{2+} (expressed in terms of total cell volume) is stored in the ER. The value of c_2 comes from our studies of RBL cells (T.M., D. Holowka, and L.S., unpublished data). The maximal transport rate into the ER was 5×10^{-18} mol of Ca^{2+} per s per cell. For a cell volume of 1 pl, this rate corresponds to 5 μM Ca^{2+}/s.

The production of $InsP_3$ depends on the catalytic activity of PLC, which is controlled by a cell-surface receptor, most likely through a G protein. A strong stimulatory effect of Ca^{2+} on PLC activity has been found in some studies (9, 10) and weaker stimulation in others (12, 14). We assume that PLC activity depends both on the degree of stimulation of the receptor and on the Ca^{2+} level. The rate of formation of $InsP_3$, denoted by k_+, is then taken to be

$$k_+ = c_4 R\, g, \qquad [4]$$

where R is the degree of receptor-dependent activation ($0 < R < 1$), and g is the modulatory effect of cytosolic Ca^{2+}. We assume that g is given by a simple binding expression,

$$g = x/(x + K_3). \qquad [5]$$

K_3 and c_4 have not been experimentally determined. However, assumed values of 1 μM and 1 μM/s lead to $InsP_3$ levels in the range (10–100 nM) expected for stimulated cells (1, 9). The rate of destruction of $InsP_3$, denoted by k_-, is given by

$$k_- = c_5 y. \qquad [6]$$

For RBL cells, c_5 is 2 s^{-1} (T.M., J. Critchfield, and L.S., unpublished results).

Relationship Between Steady-State $InsP_3$ and Ca^{2+} Levels. We can now determine how the concentration of $InsP_3$ (y) depends on the concentration of cytosolic Ca^{2+} (x), and vice versa. The rate of change of the cytosolic Ca^{2+} level is given by

$$dx/dt = J_1 - J_2 = c_1[y^3/(K_1 + y)^3]z - J_2, \qquad [7]$$

and the rate of change of $InsP_3$ concentration by

$$dy/dt = k_+ - k_- = c_4 R[x/(x + K_3)] - c_5 y. \qquad [8]$$

Consider this system at steady-state ($dx/dt = 0$ and $dy/dt = 0$) for constant $x + z$. Suppose that the $InsP_3$ concentration is clamped (held constant at a fixed value). The resulting dependence of cytosolic Ca^{2+} on [$InsP_3$] is shown in Fig. 2*a*. The cytosolic Ca^{2+} level rises steeply above 15 nM $InsP_3$ because the cooperative opening of calcium channels overwhelms the capacity of the Ca^{2+}-ATPase to pump released Ca^{2+} back into the ER. Alternatively, suppose that the cytosolic Ca^{2+} level is clamped. As shown in Fig. 2*b*, the level of $InsP_3$ depends on both the degree of receptor stimulation and the cytosolic Ca^{2+} concentration. Ca^{2+} in the 0.1–10 μM range increases the $InsP_3$ level by activating PLC.

Cooperativity and Positive Feedback Lead to Bistability. Let us now allow the concentrations of both $InsP_3$ and cytosolic Ca^{2+} to vary freely. In Fig. 3, the outcome is visualized by superimposing the [$InsP_3$] versus [Ca^{2+}] curves of Fig. 2*b* on the [Ca^{2+}] versus [$InsP_3$] curve of Fig. 2*a*. Permissible values of the concentrations of the two messengers at steady-state are given by the crosspoints. At a low degree of receptor activation, only a single value of [Ca^{2+}] (about 10^{-7} M) is allowed. Likewise, only one value (about 10^{-4} M) is possible at a high degree of receptor activation. However, there are three crosspoints at an intermediate level of receptor activation. The middle crosspoint is unstable; a small fluctuation will drive the system to one of the other two crosspoints. Thus, at a medium degree of PLC activation, two pairs of concentrations of cytosolic Ca^{2+} and $InsP_3$ are permitted. In other words, the system is *bistable* under these conditions. Calculations show that cooperativity alone or positive feedback alone do not give rise to bistability.

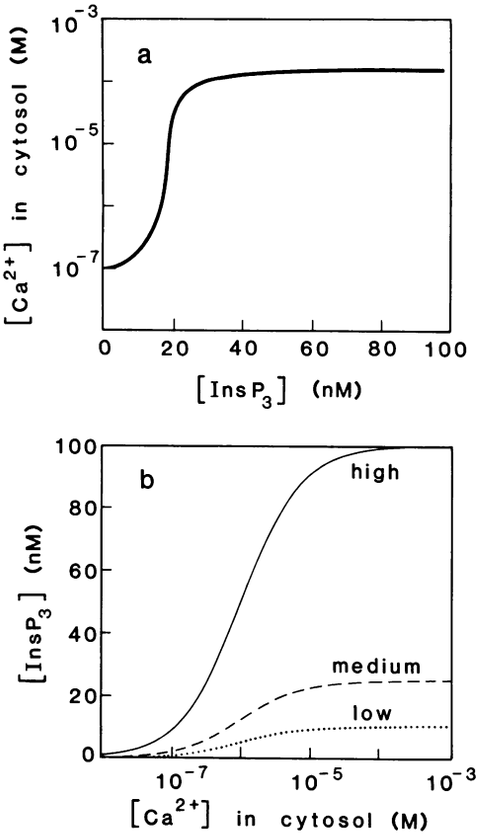

FIG. 2. Relationship between the concentrations of the crosscoupled messengers for the system depicted in Fig. 1. The rates of individual steps are given in the text. InsP_3 releases Ca^{2+} from ER stores, and cytosolic Ca^{2+} stimulates the production of InsP_3. The curves shown here also take into account the hydrolysis of InsP_3 and the pumping of Ca^{2+} back into the ER. Mitochondrial uptake of Ca^{2+}, extrusion of Ca^{2+} by plasma membrane transporters, and calcium buffering by the cytosol are not included. Inclusion of buffering would change the scale but not the pattern of these relationships. (*a*) The InsP_3 concentration is held constant at a series of values. Cytosolic [Ca^{2+}] is plotted versus clamped [InsP_3] for a steady-state in which InsP_3-induced calcium release from the ER is equal to ATP-driven calcium uptake. (*b*) The cytosolic level of Ca^{2+} is held constant at a series of values. [InsP_3] is plotted versus the clamped Ca^{2+} level for a steady-state in which InsP_3 production by PLC is equal to InsP_3 destruction. This steady-state depends on the degree of receptor stimulation (*R* in Eq. **4**) of PLC and the cytosolic Ca^{2+} level. Low, medium, and high refer to *R* values of 0.02, 0.05, and 0.2, respectively.

The Calcium Level Switches at a Threshold PLC Activation. The kinetics of transitions from the basal to the stimulated state are shown in Fig. 4. The cytosolic Ca^{2+} level stays close to 10^{-7} M, the basal value, for PLC activities up to ≈13% of maximal level. At this threshold, the system abruptly switches to the other stable state, in which the cytosolic Ca^{2+} level is near 10^{-4} M. The transition to the stimulated level is faster for higher degrees of PLC activation. However, the steady-state Ca^{2+} level is nearly independent of the degree of activation once the threshold is passed. Thus, a system with a sharply defined threshold arises from the positive interaction of crosscoupled messengers.

Depletion and Slow Repletion of the ER Calcium Store Lead to Spiking. The system depicted in Fig. 1 is bistable but cannot undergo spiking. Stimulation of PLC above the threshold leads to a persistently elevated calcium level. The cell requires additional processes to generate periodic calcium spikes. Moreover, the predicted cytosolic Ca^{2+} level of about 10^{-4} M for the stimulated state is much higher than occurs physiologically. The cytosolic Ca^{2+} level of cells,

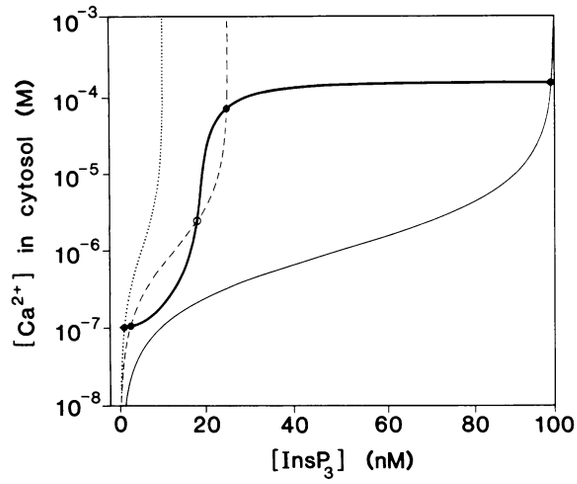

FIG. 3. Permissible values of InsP_3 and cytosolic Ca^{2+} when both are free to vary. The curves of Fig. 2*b* were rotated and superimposed on the curve of Fig. 2*a*. The crosspoints of these curves give values of [InsP_3] and [Ca^{2+}] at which the system is at steady-state. Crosspoints denoted by filled circles are stable, whereas the crosspoint denoted by an open circle is unstable.

even when stimulated, is kept below several micromolar by several transport processes. Mitochondria sequester Ca^{2+} when the cytosolic level is higher than about 0.6 μM (15). Calcium is also pumped out of cells by two types of transporters in the plasma membrane, the Ca^{2+}-ATPase and the sodium–calcium antiporter (2). The kinetics of mitochondrial uptake of calcium and their high capacity for calcium lead us to believe that mitochondrial uptake is quantitatively more important than extrusion across the plasma membrane in accounting for spiking. However, this is an open question that deserves experimental study. The major import pathway into mitochondria is a Ca^{2+} uniporter that is driven by the proton-motive force (15). The rate of entry of Ca^{2+} depends on the 3.3 power of the cytosolic Ca^{2+} concentration (for cytosolic levels up to several micromolar). The rate of efflux, in contrast, is essentially independent of the mitochondrial calcium level (15). The contributions of mitochondrial cal-

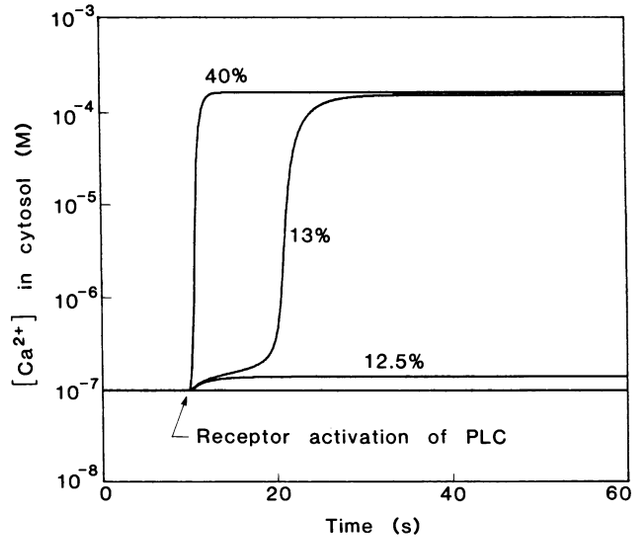

FIG. 4. Bistability of the cytosolic calcium level. The dependence of cytosolic [Ca^{2+}] on the degree of activation of PLC is shown for the system depicted in Figs. 1 and 2. Cytosolic [Ca^{2+}] remains nearly at the basal level (10^{-7} M) until PLC is activated above a threshold. The differential equations given in the text were solved numerically to generate this plot and those of Figs. 5 and 6.

cium influx and efflux are then added to Eq. 7 for the rate of change of the cytosolic Ca^{2+} level to give

$$dx/dt = J_1 - J_2 - c_6(x/c_7)^{3.3} + c_6, \quad [9]$$

where c_6 is 0.5 μM/s (15) and c_7 is 0.6 μM (the mitochondrial setpoint at which efflux is equal to influx).

The inclusion of mitochondrial sequestration has a striking effect on the time-dependence of the cytosolic calcium level. As shown in Fig. 5, spiking occurs when PLC is stimulated beyond a threshold. Spiking becomes more frequent with increasing stimulation of the receptor. The interval between spikes decreases from 145 to 53 to 24 s when the degree of receptor stimulation increases from 14 to 28.5 to 42% maximal. Thus, a 3-fold increase in receptor stimulation leads to a 6-fold increase in spike frequency. The basal and peak calcium levels change little over this range of receptor stimulation. Mitochondrial sequestration clips the cytosolic Ca^{2+} level at $\approx$1.5 μM. At 44.5% stimulation and above, spiking is rapidly damped because the ER channels are kept permanently open by the high level of $InsP_3$. This results in a persistently elevated cytosolic Ca^{2+} level of 0.6 μM, the mitochondrial setpoint.

The contributions of the processes included in Eqs. 8 and 9 to calcium spiking can be appreciated by examining the time-dependence of the concentrations of cytosolic Ca^{2+} (x) and $InsP_3$ (y), the crosscoupled messengers, and that of Ca^{2+} in the ER (z). Spiking can be divided into three phases (Fig. 6). In phase I, cooperativity and positive feedback are dominant. The highly cooperative $InsP_3$-induced release of Ca^{2+} from the ER is reinforced by enhanced production of $InsP_3$ resulting from stimulation of PLC by Ca^{2+}. The positive interplay of these messengers accounts for the steep

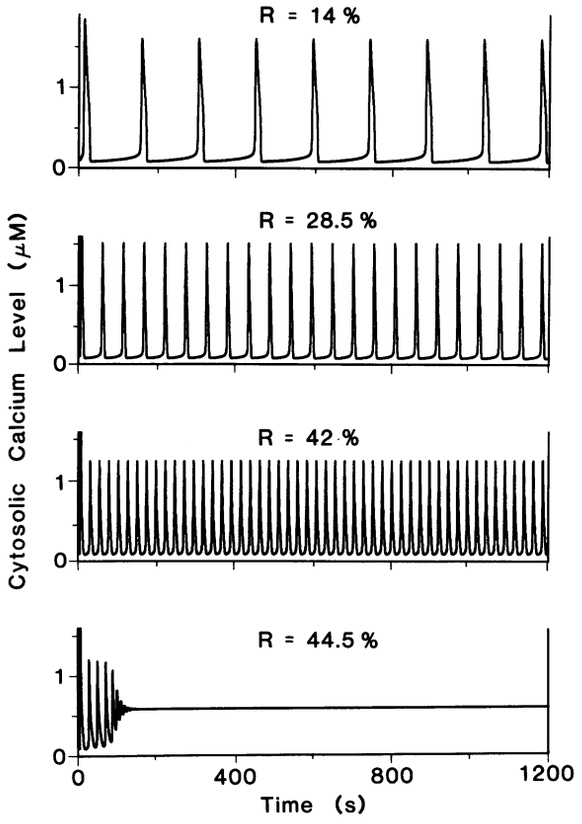

FIG. 5. Dependence of the frequency of calcium spiking on the degree of activation of PLC (expressed in terms of percent of maximal receptor-triggered stimulation, R). The time-dependence of cytosolic $[Ca^{2+}]$ was calculated for the system depicted in Fig. 4 with mitochondrial but not plasma membrane transport of Ca^{2+}.

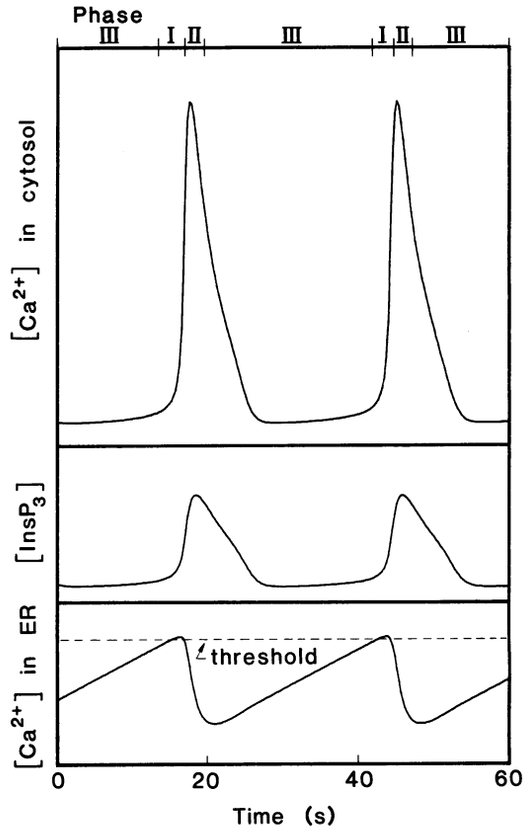

FIG. 6. Relationship between the levels of Ca^{2+} in the cytosol, $[InsP_3]$, and $[Ca^{2+}]$ in the ER under spiking conditions. The full vertical scales are 1.5 μM, 160 nM, and 27 μM, respectively.

rising portion of the spike. In phase II, mitochondrial uptake of Ca^{2+} is dominant. The Ca^{2+} level in the ER becomes depleted because the $InsP_3$-gated channels are open. The lowering of cytosolic Ca^{2+} in phase II leads to a decrease in the level of $InsP_3$ and a consequent closure of ER calcium channels. The ER Ca^{2+}-ATPase can now replenish the calcium store of the ER at the expense of the mitochondrial store. The ER has higher affinity for Ca^{2+} than do mitochondria. This repletion of the ER store dominates phase III. When the level of Ca^{2+} in the ER rises above a threshold, the cytosolic Ca^{2+} level increases swiftly to begin a new spike. Cooperativity and positive feedback once again give phase I. The variation of ER $[Ca^{2+}]$ is the *sawtooth parameter* that determines the frequency of spiking.

Proposed Tests of the Model. We have focused on only a few of many possible interactions between components of the inositol phospholipid cascade and calcium transport systems. The situation in the cell is much more complex than represented in our model. The particular processes chosen by us should be regarded as working hypotheses. Determination of the calcium dependence of PLC activity in intact cells is especially important. One approach is to clamp the cytosolic calcium level in the 50–500 nM range using extracellular Ca^{2+}-EGTA buffers and a calcium ionophore such as A23187. The level of $[^3H]$-$InsP_3$ in cells containing radiolabeled phosphatidylinositol 4,5-bisphosphate would then be measured as a function of the degree of receptor activation and the cytosolic calcium level. A second key feature of the model, depletion due to mitochondrial uptake of calcium, could be tested by specifically inhibiting this transport process with ruthenium red or by using an uncoupler that dissipates the proton-motive force across the inner mitochondrial membrane. Blockage of calcium uptake by mitochondria should prevent calcium spiking; instead, a sustained rise

in cytosolic calcium level should occur when such cells are stimulated. The sodium–calcium antiporter in the plasma membrane may complement mitochondrial uptake in lowering the cytosolic calcium level. The contribution of this antiporter to spiking can be assessed by inhibiting it using extracellular Li^+ in place of Na^+. Hormone-stimulated Ca^{2+}-influx across the plasma membrane could increase the frequency of spiking by shortening the time required for calcium loading of the ER. The significance of this process could be tested by measuring the frequency of spiking as a function of the extracellular calcium level.

Biological Significance of Periodic Calcium Spiking. The occurrence of periodic calcium spiking in a wide range of cell types and excitation processes suggests that it plays a fundamental role in transduction pathways mediated by changes in the cytosolic calcium level. The fidelity of signal transmission is increased by spiking because the resulting peak calcium levels are much higher than those produced by most spontaneous fluctuations. Furthermore, the deleterious effects of sustained elevations of calcium level can be avoided by spiking. The existence of a threshold for spiking ensures that a low degree of activation of PLC does not raise the cytosolic calcium level. A cell can ignore subthreshold stimuli or noise induced by components of the transduction chain. The rare fluctuation that triggers a single spike is unlikely to persist and lead to a train of spikes.

Cooperatively activated calcium-binding proteins, such as calmodulin, are well suited for detecting calcium spikes because they can be designed to be switched on only at spike peaks. Furthermore, effector systems can be selectively tuned to respond to particular spike frequencies (FM transmission and detection) (1, 16). A single messenger such as calcium could excite different effector systems depending on the spike frequency. One can also envision a calcium-sensitive effector system that is activated by multiple successive spikes and not by a solitary spike. Calcium spikes could also serve as an internal timing device and establish a cellular clock.

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