Control of calcium oscillations by membrane fluxes

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It is known that Ca$^{2+}$ influx plays an important role in the modulation of inositol trisphosphate-generated Ca$^{2+}$ oscillations, but controversy over the mechanisms underlying these effects exists. In addition, the effects of blocking membrane transport or reducing Ca$^{2+}$ entry vary from one cell type to another; in some cell types oscillations persist in the absence of Ca$^{2+}$ entry (although their frequency is affected), whereas in other cell types oscillations depend on Ca$^{2+}$ entry. We present theoretical and experimental evidence that membrane transport can control oscillations by controlling the total amount of Ca$^{2+}$ in the cell (the Ca$^{2+}$ load). Our model predicts that the cell can be balanced at a point where small changes in the Ca$^{2+}$ load can move the cell into or out of oscillatory regions, resulting in the appearance or disappearance of oscillations. Our theoretical predictions are verified by experimental results from HEK293 cells. We predict that the role of Ca$^{2+}$ influx during an oscillation is to replenish the Ca$^{2+}$ load of the cell. Despite this prediction, even during the peak of an oscillation the cell or the endoplasmic reticulum may not be measurably depleted of Ca$^{2+}$.

In response to an increased concentration of inositol trisphosphate (IP$_3$), oscillations in the concentration of free intracellular calcium (Ca$^{2+}$) occur in many cell types and are important for the control of many cellular functions (1–4). In nonexcitable cells, such as epithelial cells, these oscillations occur as the result of Ca$^{2+}$ flux into and out of the endoplasmic reticulum (ER). However, although the oscillations result from the cycling of Ca$^{2+}$ between the ER and the cytoplasm, the transport of Ca$^{2+}$ across the cell membrane can have a dramatic effect on these oscillations.

Although Ca$^{2+}$ influx is known to be important, disagreement exists, first, over the mechanisms by which Ca$^{2+}$ influx is modulated during a Ca$^{2+}$ oscillation, and, second, over the role played by Ca$^{2+}$ influx. The capacitative entry hypothesis proposes that depletion of ER Ca$^{2+}$ causes enhanced entry of Ca$^{2+}$ across the plasma membrane (5–9). Often, although not necessarily, in this scenario, Ca$^{2+}$ entry is necessary for the refilling of the ER, and thus oscillation frequency is controlled by the refilling time (10, 11). Although capacitative entry is certainly an important factor during the response to a maximal stimulus, other investigators have pointed to a lack of direct evidence that it plays an important role during smaller stimuli that generate oscillatory behavior (12). They maintain that Ca$^{2+}$ influx, under these conditions, is controlled by a noncapacitative pathway involving arachidonic acid and that the purpose of the influx is to increase the likelihood that low levels of IP$_3$ will induce Ca$^{2+}$ release from internal stores (13). This controversy is complicated by the fact that, in some cell types, Ca$^{2+}$ oscillations persist in the absence of Ca$^{2+}$ influx (14–16), whereas in other cell types, oscillations depend absolutely on influx (17, 18). Even in a single cell type, the effect of Ca$^{2+}$ influx on agonist-induced oscillations varies according to the agonist (19), and the effect of removal of extracellular Ca$^{2+}$ depends on when it is removed (17).

Using a combination of theoretical and experimental work, we attempt to resolve this controversy by studying how Ca$^{2+}$ transport across the cell membrane can affect ER-based oscillations. We conclude that small changes in Ca$^{2+}$ load can have a large impact on Ca$^{2+}$ oscillations. By Ca$^{2+}$ load we mean the total amount of Ca$^{2+}$ in the cell, including the ER, the cytoplasm, and the Ca$^{2+}$ buffers. (For the purposes of the model here, we neglect mitochondrial Ca$^{2+}$, but its inclusion would have no qualitative effect on our results.) The amount of Ca$^{2+}$ in the ER is directly related to the Ca$^{2+}$ load, and thus our model predicts that Ca$^{2+}$ oscillations will also be highly sensitive to ER Ca$^{2+}$ concentration.

Our results are independent of the mechanism by which Ca$^{2+}$ influx is controlled and thus cannot be used to distinguish between capacitative entry or noncapacitative, arachidonic acid-dependent entry, but they provide a way in which seemingly contradictory experimental results can be understood. In addition, they show that direct control of Ca$^{2+}$ influx by the cytosolic or ER Ca$^{2+}$ concentration is not necessary for Ca$^{2+}$ influx to play a crucial role in the control of oscillations. Finally, our results do not depend on the precise details of the model, but they occur in at least four different models of Ca$^{2+}$ oscillations.

The Model

Our model for Ca$^{2+}$ oscillations is based on a dynamic model of the IP$_3$ receptor (IPR) (20) and assumes that oscillations in [IP$_3$] are not necessary for the generation of Ca$^{2+}$ oscillations. A schematic diagram of the cell is given in Fig. 1. From there we see that the conservation equations for Ca$^{2+}$ are

\[
\frac{dc}{dt} = J_{\text{IPR}} - J_{\text{serca}} + \delta (U_{\text{in}} - U_{\text{pm}}),
\]

\[
\frac{dc_e}{dt} = \gamma J_{\text{serca}} - J_{\text{IPR}},
\]

where $c$ and $c_e$ denote [Ca$^{2+}$] in the cytoplasm and ER, respectively. \(\gamma\) is the cytoplasmic-to-ER volume ratio, and $\delta$ is a dimensionless parameter that controls the relative magnitudes of membrane and ER fluxes without changing the steady-state concentrations.

Calcium release through the IPR is described by the model of ref. 20, slightly modified for the context of a whole-cell model. Thus,

\[ J_{\text{IPR}} = [k_f (0.1O + 0.94) + g_1](c_e - c), \]

where $g_1$ models a constant background leak from the ER at zero [IP$_3$]. $O$ and $A$ denote the probability the IPR is in the open and activated state, respectively, and are governed by differential equations with parameters determined by fitting to experimental data from the type II hepatocyte IPR (21).

Calcium pumping into the ER ($J_{\text{serca}}$) and extrusion across the plasma membrane ($J_{\text{pm}}$) are modeled by

\[ J_{\text{serca}} = \frac{V_c c}{K_c + c} \left( \frac{1}{c_e} \right), \quad J_{\text{pm}} = \frac{V c_e^2}{K_p + c} c. \]
These expressions are the ones used by ref. 22 and are discussed in detail there. Ca\(^{2+}\) entry is modeled as a linear, increasing function of [IP\(_3\)] (\(p\)), and thus

\[
J_{in} = \alpha_1 + \alpha_2 p,
\]

where \(\alpha_1\) and \(\alpha_2\) are constants. This form for \(J_{in}\) is entirely arbitrary; we know that \(J_{in}\) must be an increasing function of \(p\) but do not know the exact relationship. Thus, we just choose the simplest possible form. Using a more complex function for \(J_{in}\) makes no qualitative difference to the model results.

Buffering is taken into account by assuming each flux to be an effective flux, scaled by the effects of fast linear buffers. Inclusion of slower nonlinear buffers will not alter the qualitative results of our analysis.

The parameter values are given in the legend to Fig. 2.

**Analysis of the Model**

Introduction of the new variable, \(c_1 = c + (1/\gamma)c_e\) gives the system

\[
\frac{dc}{dt} = \delta J_{in} - J_{pm},
\]

and

\[
\frac{dc}{dt} = J_{IPR} - J_{sérica} + \delta J_{in} - J_{pm}.
\]

The new variable \(c_1\) is the total number of moles of Ca\(^{2+}\) in the cell divided by the cytoplasmic volume. We shall call \(c_1\) the Ca\(^{2+}\) load of the cell. When \(\delta\) is small, \(c_1\) can change only slowly, and in the limit, as \(\delta \to 0\), the cell becomes pseudosteady; the solution is trying to follow the oscillatory line as \(t \to \infty\). Thus, in response to an increase in \([IP_3]\), the model cell exhibits transient pseudosteady oscillations that tend to a stable steady-state limit cycle, in which the oscillation is a degenerate bursting oscillation having only a single peak during each bursting phase. This slow modulation of the oscillations is caused by a gradual decrease in \(c_1\) and so the speed with which the model reaches the steady-state oscillation is governed by the value of \(\delta\), which controls the speed at which \(c_1\) reaches a steady-state limit cycle.

If an open cell exhibits Ca\(^{2+}\) oscillations, how will these oscillations be affected if membrane transport is stopped? This is most easily studied with the bifurcation diagram shown in Fig. 3. The loci of HC and SNP bifurcations as \(p\) and \(c_1\) vary in the closed-cell model are shown by the red curves. Thus, for each value of \(p\), the closed-cell model will exhibit oscillations only when \(c_1\) lies between these two curves. Superimposed on this we plot bifurcation diagrams of the open-cell model (broken curves), plotting the maximum and minimum of \(c_1\) over an oscillation as a function of \(p\). For each value of \(p\) where steady oscillations exist in the open-cell model, \(c_1\) moves between the appropriate broken curves. (For instance, when \(\delta = 0.1\), the
However, if the block occurs later, after closed-cell models gets wider (region E), which stable-steady oscillations coexist in the open-cell and the open-cell sits in region D, blockage of membrane transport will not limit as $t$ has decreased past $c_0$, the open-cell oscillations get progressively reduced to zero, i.e., membrane transport is shut off, $c_1$ will now be held constant at point A and will not lie between the HC and SNP curves. Hence, the closed-cell model cannot oscillate and the oscillations stop. To restore the oscillations, $c_1$ could be increased until it crosses the HC curve (point B). Alternatively, oscillations can be restored by increasing $p$ (point C).

When $p = 40$ and $\delta = 0.01$, we see that stable oscillations coexist in both the open-cell and closed-cell model, but for only a relatively small region (region D). If $p$ and $c_1$ are such that the cell sits in region D, blockage of membrane transport will not eliminate $Ca^{2+}$ oscillations. When $\delta = 0.001$, i.e., when membrane transport in the open-cell model is very slow, the open-cell bifurcation curves (the broken lines) begin to superimpose on the HC curve of the closed-cell model, thus showing how, in the limit as $\delta \to 0$, the open-cell oscillations get progressively restricted to planes of constant $c_1$. In addition, the $p$ interval for which stable-steady oscillations coexist in the open-cell and the closed-cell models gets wider (region E).

From Fig. 2 we see that, immediately after an increase in $[IP_3]$, the open-cell model exhibits oscillations with a gradually decreasing baseline and with a decreasing average $c_1$. This finding suggests that the effects of blocking membrane transport will depend on the timing of the block. If the block occurs soon after, or before, the increase in $[IP_3]$, oscillations can continue because $c_1$ has not yet fallen far enough to make oscillations impossible. However, if the block occurs later, after $c_1$ has decreased past HC, then the block will be expected to eliminate oscillations.

Influx of $Ca^{2+}$ from outside the cell is known to affect the frequency of $Ca^{2+}$ oscillations and waves. We investigate this in the open-cell model by varying the background rate of $Ca^{2+}$ influx, $\alpha_1$. In Fig. 4 we plot lines in the $\alpha_1 - p$ plane that separate regions where $Ca^{2+}$ oscillations occur from regions where they do not. For a fixed $p$, an increase in $Ca^{2+}$ influx will lead to oscillations or to a decrease in the oscillation period. Similarly, for a fixed $\alpha_1$, an increase in $p$ will have the same effect. This occurs even when membrane transport is slow ($\delta = 0.001$). Oscillations can be eliminated by a decrease in influx but can then be restored by increasing $p$.

**Physiological Interpretation of the Analysis**

A model cell can fire a $Ca^{2+}$ spike only if its $Ca^{2+}$ load ($c_i$) is above threshold. This is because enough cytosolic $Ca^{2+}$ must be present to activate the $IP_3$ receptor, and enough ER $Ca^{2+}$ must be available to provide sufficient driving force for efflux from the ER. Once $c_1$ drops below threshold, these conditions are violated and the cell cannot fire a $Ca^{2+}$ spike.

At rest, the model cell has a high resting $Ca^{2+}$ load ($c_i$). On agonist stimulation, the rise in cytosolic $Ca^{2+}$ causes a net loss of $Ca^{2+}$ from the cell (as it is removed by the plasma membrane pumps), even though the influx of $Ca^{2+}$ has been increased. Thus, $c_1$ gradually declines, resulting in oscillations with a gradually decreasing baseline. If membrane transport is blocked during this phase of the response, $c_1$ is still high enough to maintain oscillations, which would then occur purely by recycling $Ca^{2+}$ to and from the ER.

Eventually, $c_1$ declines to a new steady-state average level. Now, at the peak of each oscillation, the high cytosolic concentration causes a net loss of $Ca^{2+}$ from the cell. The $Ca^{2+}$ load thus drops below threshold, and the cell cannot fire another spike until this $Ca^{2+}$ loss is restored by $Ca^{2+}$ influx. Because each oscillation involves the loss and reuptake of $Ca^{2+}$ from the cell (possibly only a small amount), these oscillations depend on $Ca^{2+}$ influx and will be eliminated by blockage of membrane transport.

An increase in $Ca^{2+}$ influx will increase oscillation frequency, as it increases the rate at which the $Ca^{2+}$ lost during each spike is regained. In addition, an increase in $Ca^{2+}$ influx will increase the average steady-state $Ca^{2+}$ load, which will also increase oscillation frequency, even when the oscillations are not dependent on $Ca^{2+}$ influx. A decrease in $Ca^{2+}$ influx can eliminate oscillations, which can then be restored by an increase in $[IP_3]$.

When membrane transport is slow enough, $Ca^{2+}$ oscillations can persist in the absence of membrane transport, although their frequency is affected by $Ca^{2+}$ influx. A closed cell needs a higher $Ca^{2+}$ load to support an oscillation than does an open cell. In the closed cell, because the $Ca^{2+}$ load is constant, oscillations are possible only if the $Ca^{2+}$ load is...
always over threshold. In the open cell this constraint is relaxed. For a large part of the oscillatory cycle, the Ca$^{2+}$ load can be below threshold. It is only when Ca$^{2+}$ influx from the outside drives the Ca$^{2+}$ load over threshold that a spike occurs. Some of the Ca$^{2+}$ released from the ER is then pumped out of the cell, the Ca$^{2+}$ load drops below threshold, and the cycle repeats. Thus, when averaged over one cycle, the Ca$^{2+}$ load is lower for the open cell than for the closed cell. For open-cell oscillations, Ca$^{2+}$ influx serves to increase the Ca$^{2+}$ load to a level where another oscillation can occur, and an increase in Ca$^{2+}$ influx increases the oscillation frequency. However, although it is cell refilling that is governing the oscillation period, the amount of Ca$^{2+}$ actually lost from the cell during each oscillation can be small and not easily detectable.

From our analysis we predict the following:

**Prediction 1.** Blocking membrane transport can eliminate Ca$^{2+}$ oscillations. An increase in the Ca$^{2+}$ load of the cell will restore oscillations that have been eliminated by the block.

**Prediction 2.** The effects of blocking membrane transport will depend on the timing of the block. If the block occurs soon after, or before, the increase of [IP$_3$]$_i$, it will not eliminate oscillations, whereas if it occurs after the cell has reached a steady-state oscillation, it will eliminate the oscillations.

**Experimental Verification**

We tested our predictions experimentally in the following way. HEK293 cells were stably transfected with the m3 human muscarinic receptor and loaded with fluo-4. They were imaged on a TILL Photonics system with a monochromator (TILL Photonics, Planegg, Germany) and a progressive line-scan digital camera (Imago, Scientific Instruments, Madison, WI). The cells were stimulated with 0.5 mM carbamylcholine (CCh) to induce Ca$^{2+}$ oscillations, then 1 mM La$^{3+}$ was added to block both Ca$^{2+}$ entry and the plasma membrane Ca$^{2+}$ pump (29–33). Fig. 5 shows the effects of the addition of La$^{3+}$ at two different times: after steady oscillations had appeared (Upper) and immediately before the addition of CCh (Lower). As predicted, oscillations were eliminated in the first case but not in the second (20 cells in four separate experiments). In the first case, the effect of La$^{3+}$ was reversible. This result is similar to previous results showing that removal of extracellular Ca$^{2+}$ before application of CCh to pancreatic acinar cells did not prevent oscillations, whereas removal of extracellular Ca$^{2+}$ from a cell exhibiting Ca$^{2+}$ oscillations terminated oscillations (17).

In a second set of experiments, the cells were also loaded with caged Ca$^{2+}$. Then, after the addition of La$^{3+}$, intracellular Ca$^{2+}$ was increased by uncaging the Ca$^{3+}$ with the “strobe” mode on the TILL flash unit. This mode releases Ca$^{2+}$ by brief low-energy flashes of UV light at 12 Hz. This strobing typically lasted for ~1–1.5 min (Fig. 6). Then the La$^{3+}$ was washed out, still in the presence of 0.5 mM CCh. A typical result (from seven cells in three separate experiments) is shown in Fig. 6. On addition of CCh, the cell exhibited Ca$^{2+}$ oscillations, which terminated on blockage of membrane transport, even though the Ca$^{2+}$ load of the cell remained unchanged by the addition of La$^{3+}$. Thus, it is not the Ca$^{2+}$ load alone that is controlling the oscillations, it is the combination of the Ca$^{2+}$ load and the transport across the plasma membrane. An increase in $c_1$ by the strobe then leads to the resumption of Ca$^{2+}$ oscillations, which persist even when the strobe is turned off. Thus, once $c_1$ is increased sufficiently, Ca$^{2+}$ oscillations persist even in the absence of membrane transport, as predicted by the model. When membrane transport is restored by the removal of La$^{3+}$, the oscillations continue.

Application of the strobe in the absence of Ca$^{2+}$, but the presence of La$^{3+}$, resulted in a small increase in resting [Ca$^{2+}$] (Fig. 6, red line). On removal of the strobe, [Ca$^{2+}$] remained unchanged, because the La$^{3+}$ prevented it from leaving the cell; on removal of the La$^{3+}$, [Ca$^{2+}$] returned to baseline. Application of the strobe in the absence of caged Ca$^{2+}$ has no effect on oscillations (34).

**Discussion**

Our results suggest that membrane transport of Ca$^{2+}$ controls oscillations by controlling the Ca$^{2+}$ load of the cell. It is already well known that Ca$^{2+}$ influx affects oscillation frequency in many cell types (10, 13, 35–37). In *Xenopus* oocytes, which have a very small surface-to-volume ratio and thus a small $\delta$, Ca$^{2+}$ oscillations persist in the absence of external Ca$^{2+}$, as found in the...
model. Nevertheless, an increase in Ca\textsuperscript{2+} influx increases oscillation frequency and wave speed (16, 35). In pancreatic acinar cells application of acetylcholine gives rise to Ca\textsuperscript{2+} oscillations, which disappear on reduction of external Ca\textsuperscript{2+}. However, these oscillations reappear when the concentration of acetylcholine is increased (38), as expected from the results in Fig. 4. The effects of Ca\textsuperscript{2+} load on oscillations and waves have also been well characterized in cardiac cells (39–41).

Our model is also consistent with the results of ref. 42, which showed that blockage of Ca\textsuperscript{2+} influx during the interspike period eliminated further spikes, whereas, if the block occurred during a spike, the spike occurred as usual. According to our model, this is because only a small amount of Ca\textsuperscript{2+} influx during the interspike period is needed to push the Ca\textsuperscript{2+} load of the cell over threshold, whereas the majority of the Ca\textsuperscript{2+} in a spike is released from the ER.

We have shown that it is not the Ca\textsuperscript{2+} load per se that is the controlling factor, but it is rather the interplay between Ca\textsuperscript{2+} load and membrane transport. In HEK293 cells, addition of CCh stimulates Ca\textsuperscript{2+} oscillations with a gradually decreasing baseline (Fig. 6). If membrane transport is then blocked, the oscillations reappear when the concentration of acetylcholine is increased (38), as expected from the results in Fig. 4. The effects of blocking membrane transport depend on the timing of the block. If the block occurs while the Ca\textsuperscript{2+} load is high, i.e., before application of agonist, an increase in [IP\textsubscript{3}] will cause oscillations, whereas if the block occurs later, after the Ca\textsuperscript{2+} load has declined, agonist-induced oscillations will be eliminated (Fig. 5). Thus, our model can also explain previous puzzling results of ref. 17, which showed that removal of extracellular Ca\textsuperscript{2+} before the application of CCh did not eliminate oscillations, whereas, if Ca\textsuperscript{2+} oscillations had already developed, they were eliminated by the removal of extracellular Ca\textsuperscript{2+}.

That small changes in Ca\textsuperscript{2+} load can have large effects on oscillations suggests a possible resolution of the controversy over the role of Ca\textsuperscript{2+} entry. Since experimental data have been unable to show conclusively that the ER is significantly depleted during an oscillation (43), it has been argued that ER depletion, and hence capacitative Ca\textsuperscript{2+} entry, does not play a significant role (12). In contrast, our model shows that cell depletion (i.e., a decrease in cytosolic and ER Ca\textsuperscript{2+}) can play a crucial role, even when depletion is so small as to be unmeasurable. Our analysis also provides a consistent framework for understanding why removal of extracellular Ca\textsuperscript{2+} has such different effects, depending on the cell type, and it can explain why the timing of removal is so important (17, 42).

The same qualitative behavior occurs in at least three other models of Ca\textsuperscript{2+} oscillations: the Li–Rinzel model (44), and modified versions of the Atri model (45) and the LeBeau model (38) (computations not shown), all of which have different models for the IP\textsubscript{3} receptor and Ca\textsuperscript{2+} pumps. This finding suggests that our predictions do not depend on the precise model details, but they are instead a generic feature of models that have the structure shown in Fig. 1. We hypothesize that every model to follow this basic structure will exhibit the same behavior, no matter what the exact details are of the various pumps and release processes.

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