Inositol 1,4,5-Trisphosphate-dependent Oscillations of Luminal [Ca^{2+}] in Permeabilized HSY Cells*

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Oscillations in intracellular Ca^{2+} concentration ([Ca^{2+}]_{i}) are thought to play an important role in phosphoinositide-linked Ca^{2+} signaling events. We demonstrate corresponding inositol 1,4,5-trisphosphate (IP_{3})-dependent oscillations of Ca^{2+} concentration within the lumen of the IP_{3}-sensitive stores ([Ca^{2+}]_{i}) of saponin-permeabilized HSY cells by monitoring [Ca^{2+}]_{i} with the fluorescent Ca^{2+} indicator Mag-fura-2. The associated openings and closings of the IP_{3}-sensitive Ca^{2+} release channel were detected via quenching of Mag-fura-2 fluorescence due to the entry of Mn^{2+}, a Ca^{2+} surrogate, into the stores. Evidence for complimentary Ca^{2+} oscillations at the external surface of the stores was provided by the membrane-bound Ca^{2+} probe Calcium Green C_{18}. The permeabilization of saponin-treated HSY cells to macromolecules was confirmed by finding that permeabilized cells readily took up and lost (t_{1/2} ~ 46 s) a fluorescently tagged 70-kDa dextran. Our results impose a number of constraints on possible mechanisms for [Ca^{2+}]_{i} oscillations. In addition, they support recent proposals that [Ca^{2+}]_{i} oscillations arise directly from the (biphasic) effects of Ca^{2+} itself on IP_{3}-sensitive Ca^{2+} channel activity.

In many cell types, stimulation of receptors linked to phosphoinositide signaling pathways results in oscillations of intracellular calcium concentration ([Ca^{2+}]_{i}) due to the periodic release and reuptake of Ca^{2+} by intracellular stores (1–4). These oscillations often take the form of “base-line spikes” in which periodic spikes of [Ca^{2+}]_{i} rise from a base-line [Ca^{2+}]_{i}, which is close to resting levels. It has been suggested that [Ca^{2+}]_{i} oscillations provide a sort of digital encoding of the receptor-mediated signal that may have certain advantages, such as noise reduction and decreased exposure to high intracellular Ca^{2+} levels, over a continuously graded increase in [Ca^{2+}]_{i} (1–4).

Although a number of explanations have been proposed to account for [Ca^{2+}]_{i} oscillations (1–4), at present there is no clear consensus regarding the intracellular events responsible for their generation. Early suggestions included models in which these oscillations were linked to oscillations in intracellular IP_{3} concentration, and models involving interactions between Ca^{2+} release from two (or more) intracellular Ca^{2+} pools. In the former case oscillations in intracellular IP_{3} levels were proposed to arise from feedback by protein kinase C or [Ca^{2+}]_{i} itself on early steps in phosphoinositide signaling. In the latter case it was suggested that IP_{3}-dependent Ca^{2+} release from one pool induces an explosive Ca^{2+} release from another Ca^{2+}-sensitive or less IP_{3}-sensitive pool, thereby generating a spike in [Ca^{2+}]_{i}, followed by a period of reloading then a retriggering of explosive Ca^{2+} release. More recently, models incorporating the experimentally observed modulatory effects of Ca^{2+} itself on the IP_{3}-sensitive Ca^{2+} channel (5, 6) have received considerable attention (1–3, 7, 8). These experiments indicated that the open probability of the IP_{3}-sensitive channel had a bell-shaped dependence on [Ca^{2+}]_{i}, such that small increases in [Ca^{2+}]_{i} above resting levels would be expected to activate the channel, but large increases would be inhibitory. Several detailed mathematical analyses (9–11) have demonstrated that a single intracellular Ca^{2+} pool possessing IP_{3} receptors with properties derived from these (and other) empirical measurements can produce [Ca^{2+}]_{i} oscillations that closely resemble experimental observations. Thus these calculations substantiate the interesting possibility that [Ca^{2+}]_{i}, oscillations may occur solely as a result of the intrinsic properties of the IP_{3}-sensitive Ca^{2+} channel. Briefly, the underlying operation of these models is the following. A small initial rise in [Ca^{2+}]_{i}, due to IP_{3}-dependent Ca^{2+} release results in a rapid Ca^{2+}-dependent increase in IP_{3}-sensitive channel activity. This feed forward effect of Ca^{2+} on its own release generates the upstroke of a [Ca^{2+}]_{i} spike. At this high [Ca^{2+}]_{i}, a somewhat slower process of Ca^{2+} feedback inhibition then results in channel closure. Ca^{2+} reuptake into intracellular stores via the SERCA pump subsequently returns [Ca^{2+}]_{i}, to near base-line levels where Ca^{2+} feedback inhibition is slowly relieved, followed by reactivation of IP_{3}-induced Ca^{2+} release and a repetition of the [Ca^{2+}]_{i} oscillation cycle.

To date experimental studies of [Ca^{2+}]_{i} oscillations have utilized intact cells, making it difficult to definitively confirm or exclude the involvement of many intracellular processes in this phenomenon, and consequently complicating tests of proposed mechanisms. In the work presented here we extend these studies to permeabilized cells. For our experiments we have employed the HSY cells, a human salivary epithelial cell line (12) that has been found to be a useful model system for investigations of intracellular Ca^{2+} signaling events (13–15). We have previously shown (15) that the luminal calcium concentration ([Ca^{2+}]_{l}) within the IP_{3}-sensitive intracellular Ca^{2+} stores of saponin-permeabilized HSY cells can be monitored with the fluorescent Ca^{2+} indicator Mag-fura-2. Using this permeabi-
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**RESULTS AND DISCUSSION**

In order to observe \([\text{Ca}^{2+}]_{i}\) in permeabilized cells we have employed the low affinity \([\text{Ca}^{2+}]_{i}\)-sensitive fluorescent dye Mag-fura-2 (K\(_D\) for \([\text{Ca}^{2+}]_{i}\) \(\sim 53 \mu\text{M}\)) (16) which, under appropriate loading conditions, accumulates in the IP\(_3\)-sensitive stores of HSY (15) and other cells (17, 18) and can be used to monitor changes in \([\text{Ca}^{2+}]_{i}\). Preliminary experiments in our laboratory have established that single intact HSY cells loaded with the high affinity \([\text{Ca}^{2+}]_{i}\)-indicator fura-2 exhibit typical base-line spike-type \([\text{Ca}^{2+}]_{i}\) oscillations following stimulation with IP\(_3\). The ratio of Mag-fura-2 fluorescence at excitation wavelengths 344 and 360 nm, which is a direct measure of \([\text{Ca}^{2+}]_{i}\) (15–17), is seen to oscillate several times when [IP\(_3\)] is increased from 0.3 to 0.6 \(\mu\text{M}\), and to oscillate regularly when [IP\(_3\)] \(\geq 1 \mu\text{M}\). In experiments of this type we have found that oscillations in \([\text{Ca}^{2+}]_{i}\) occur in response to IP\(_3\) in approximately 50% of HSY cells treated as described here, with some variation in sensitivity to IP\(_3\) and frequency of oscillation from cell to cell. The average period of oscillation at 1 \(\mu\text{M}\) IP\(_3\) was 42.6 ± 3.0 s (n = 14). A highly statistically significant decrease in oscillation period was observed with increasing [IP\(_3\)]; the time between oscillations decreased 45 ± 13% (n = 4), 43 ± 16% (n = 4) and 42 ± 7% (n = 5) when [IP\(_3\)] was increased from 0.3 to 0.6 \(\mu\text{M}\), 0.6 to 1.0 \(\mu\text{M}\), and 1.0 to 3.0 \(\mu\text{M}\), respectively.

The oscillations in \([\text{Ca}^{2+}]_{i}\) observed in Fig. 1 have a characteristic shape that was seen in virtually all of our experiments. Each oscillation consists of an abrupt fall in the Mag-fura-2 ratio followed by a (usually incomplete) recovery, then a pause or refractory period before the beginning of the next oscillation. A similar pattern has recently been observed in measurements of \([\text{Ca}^{2+}]_{i}\) during agonist-induced \([\text{Ca}^{2+}]_{i}\) oscillations in intact cells (18, 19). In our experiments the half times for \([\text{Ca}^{2+}]_{i}\) re-

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**MATERIALS AND METHODS**

**Media—**...
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leak and recovery at 1 μM IP<sub>3</sub> were 5.7 ± 0.6 s and 7.1 ± 0.5 s, respectively (n = 14). No significant change in either of these parameters could be detected with variations of [IP<sub>3</sub>] (range 0.3–3.0 μM; n = 5 at each concentration). This latter observation suggests that the changes in oscillatory period with [IP<sub>3</sub>] documented above are mainly due to changes in the length of the refractory period between oscillations.

In order to investigate the fate of the Ca<sup>2+</sup> lost from intracellular stores during [Ca<sup>2+</sup>]<sub>L</sub> oscillations we briefly exposed Mag-fura-2-loaded, permeabilized HSY cells to the lipophilic Ca<sup>2+</sup> indicator Calcium Green C<sub>18</sub> (20). This indicator consists of a highly hydrophilic calcium-green molecule conjugated to a lipophilic alkyl chain that will insert into biological membranes with which it has direct contact. In Fig. 2A we illustrate the results of an experiment in which Mag-fura-2 fluorescence (upper trace) and Calcium Green C<sub>18</sub> fluorescence (lower trace) were monitored simultaneously in the same permeabilized HSY cell. This experiment demonstrates that each oscillation in [Ca<sup>2+</sup>]<sub>L</sub> reported by Mag-fura-2 is accompanied by a complimentary oscillation in [Ca<sup>2+</sup>]<sub>L</sub> reported by Calcium Green C<sub>18</sub>. In particular, the Calcium Green C<sub>18</sub> signal rises as [Ca<sup>2+</sup>]<sub>L</sub> falls, peaking close to the point of the [Ca<sup>2+</sup>]<sub>L</sub> minimum, then falls to base-line levels as [Ca<sup>2+</sup>]<sub>L</sub> recovers. Given these strong temporal correlations and the known properties of the two fluorescent probes the obvious explanation of this result is that the Ca<sup>2+</sup> released from the IP<sub>3</sub>-sensitive intracellular store causes an increase in [Ca<sup>2+</sup>]<sub>L</sub> close to the surface of the store ([Ca<sup>2+</sup>]<sub>L</sub>) which is, in turn, detected by the Calcium Green C<sub>18</sub> incorporated into these membranes.

In Fig. 2B we show that a similar correlation between [Ca<sup>2+</sup>]<sub>L</sub> and [Ca<sup>2+</sup>]<sub>S</sub> is seen even in the presence of 100 μM EGTA. Thus the quantity of Ca<sup>2+</sup> released during each oscillation is sufficiently large that it cannot be buffered by this concentration of EGTA, suggesting that the increase in [Ca<sup>2+</sup>]<sub>S</sub> near the IP<sub>3</sub>-sensitive channel is also quite large. In numerous previous experiments carried out in the presence of 1 mM EGTA, the application of IP<sub>3</sub> to permeabilized HSY cells consistently resulted in sustained Ca<sup>2+</sup> release rather than oscillations of [Ca<sup>2+</sup>]<sub>L</sub> (15) (experiments were carried out essentially as described here but in ICM). This sustained release was accompanied by little or no increase in [Ca<sup>2+</sup>]<sub>S</sub> as monitored by Calcium Green C<sub>18</sub> (not shown).

The loss of oscillatory behavior with increased Ca<sup>2+</sup> buffering by EGTA is consistent with the hypothesis that modulatory effects of [Ca<sup>2+</sup>]<sub>L</sub>, itself underlie the generation of [Ca<sup>2+</sup>]<sub>L</sub> oscillations. It has been suggested that Ca<sup>2+</sup>-dependent phosphorylation/dephosphorylation events may be involved in these effects (21). However, in our experiments we have found that [Ca<sup>2+</sup>]<sub>L</sub> oscillations persist in permeabilized HSY cells in the presence of either the relatively nonspecific protein kinase inhibitor staurosporine (1 μM; n = 3) or the Ca<sup>2+</sup>/calmodulin-dependent kinase inhibitor compound 5 (2 μM; n = 3) arguing against a role of protein phosphorylation in oscillatory behavior in this system.

In order to monitor the opening of the IP<sub>3</sub>-sensitive Ca<sup>2+</sup> channel directly we took advantage of earlier demonstrations that Mn<sup>2+</sup> is a substrate for the channel (15, 22–24) and of the fact that Mn<sup>2+</sup> entry can be detected by its quenching of Mag-fura-2 fluorescence. A typical experiment is shown in Fig. 3. Mag-fura-2 fluorescence measured at 344 nm excitation (the isosbestic wavelength) and the 344/360 fluorescence ratio are shown. Thus the former is an indicator of Mag-fura-2 fluoro-
FIG. 4. Permeabilization of saponin-treated HSY cells to rhodamine B-dextran (70 kDa). *Left-hand panel*, following treatment with saponin and exposure to Calcium Green C18 (see "Materials and Methods"), HSY cells were incubated with rhodamine B-dextran (0.1 mg/ml in ICM) for 5 min, after which the dextran was washed away. Calcium Green C18 and rhodamine B-dextran fluorescence were monitored via confocal microscopy as described under "Materials and Methods." Images A–E show the same field (140 μm × 140 μm) of seven HSY cells. A is a bright field image; B is a confocal image of Calcium Green C18 fluorescence; C, D, and E are confocal images of rhodamine B-dextran fluorescence taken before washing away the dextran (C), 30 s after washing away extracellular dextran (D) and 10 min after dextran removal (E). *Right-hand panel*, IP3-induced oscillations in Calcium Green C18 fluorescence in a single saponin-permeabilized HSY cell which was also confirmed to be permeabilized to rhodamine B-dextran. Intracellular Calcium Green C18 fluorescence (in arbitrary units) has been smoothed by averaging data points over a 10-s window (in order to confirm the dextran permeability of the cell the data were collected on the confocal microscope, which is significantly noisier than microfluorimetry for measurements of this type; cf. Fig. 2).

...oscillations while the latter provides a measure of [Ca\(^{2+}\)]\(_i\). At the onset of each oscillation in [Ca\(^{2+}\)]\(_i\), a relatively rapid increase in the rate of quenching of Mag-fura-2 is observed, indicating increased Mn\(^{2+}\) entry into the Mag-fura-2 compartment. Note, however, that there is a significant delay between the point of maximal Ca\(^{2+}\) release and the closing of the channel as indicated by termination of Mn\(^{2+}\)-induced fluorescence quenching (11.7 ± 2.1 s; n = 3). This observation is initially puzzling since it argues against the intuitive notion that [Ca\(^{2+}\)]\(_i\) recovery occurs as a consequence of channel closure. But as discussed below this behavior is, in fact, predicted by recently proposed models of [Ca\(^{2+}\)]\(_i\) oscillations. An obvious concern in the interpretation of the data presented above is the degree of permeabilization resulting from the procedures employed here. This issue was explored in the experiments illustrated in Fig. 4. In the *left-hand panel* of Fig. 4 we show five images of the same field of HSY cells obtained as follows. A is a bright field image of seven HSY cells treated with saponin, then exposed to Calcium Green C18 as described under "Materials and Methods." B is a confocal image of Calcium Green C18 fluorescence. Note that in two of the cells there is considerable fluorescent labeling of internal sites indicating that they are permeabilized to Calcium Green C18, while in the other five cells the dye has been largely excluded from the cell interior and a clearly delineated ring of plasma membrane labeling is seen. Next the cells were exposed to rhodamine B-dextran (70 kDa; 0.1 mg/ml). C is a confocal image of rhodamine B-dextran fluorescence taken 5 min later. The image shows incorporation of the dextran into the same two cells that were permeabilized to Calcium Green C18 in image B and exclusion from the five others. D and E are also confocal images of rhodamine B-dextran fluorescence taken 30 s and 10 min, respectively, after washing away extracellular dextran. It is clear from images C, D, and E that the two cells that are permeabilized to Calcium Green C18 also readily take up and lose rhodamine B-dextran and thus are likewise permeabilized to macromolecules (70 kDa). In experiments of this type we found that, of 119 cells showing intracellular staining with Calcium Green C18 (cf. image B), only two excluded rhodamine B-dextran. Of these 117 cells permeabilized to rhodamine B-dextran, 50% (58 cells) exhibited oscillations in Calcium Green C18 fluorescence after exposure to IP3 (right-hand panel of Fig. 4).

The degree of permeabilization to rhodamine-dextran was estimated in a subset of the cells examined above by determining the t\(_{1/2}\) for rhodamine-dextran fluorescence loss following the removal of extracellular dye (see "Materials and Methods"). In cells showing oscillations in Calcium Green C18 fluorescence in response to IP3 this t\(_{1/2}\) was 46.1 ± 7.0 s (n = 33), while in nonoscillating cells t\(_{1/2}\) was 46.3 ± 8.1 s (n = 37). Thus it is clear that there is no significant difference in dextran permeability between oscillating and nonoscillating cells.

The above experiments impose a number of constraints on possible explanations of [Ca\(^{2+}\)]\(_i\) oscillations in HSY cells. The observation that oscillations of [Ca\(^{2+}\)]\(_i\) can be observed in permeabilized cells where the containment of cytosol and the permeability barrier of the plasma membrane are largely lost argues strongly against the participation of any cellular enzymes or structures that are not a part of, or localized very close to, the IP3-sensitive Ca\(^{2+}\) stores. In addition, it indicates that the involvement of any diffusible cytosolic messengers must occur over relatively short distances and/or the localized concentrations of these messengers must be large. The possibility that oscillations in [Ca\(^{2+}\)]\(_i\), are linked to oscillations in [IP3] in HSY cells is essentially excluded. Furthermore the fact that pulsatile Ca\(^{2+}\) release persists as the Ca\(^{2+}\) content of the stores gradually falls (Fig. 1) cannot be accounted for by models that require the complete refilling of intracellular stores between oscillations. These latter observations also argue strongly against a significant role of [Ca\(^{2+}\)]\(_i\) itself in the mechanism by which oscillatory behavior is generated.

Our observations cannot definitively exclude the possibility that [Ca\(^{2+}\)]\(_i\) oscillations may involve interactions between closely associated intracellular Ca\(^{2+}\) pools. But as indicated above they do considerably limit the way in which these proposed mechanisms can operate. It is particularly interesting to note, however, that our results are quite consistent with recently proposed mechanisms for [Ca\(^{2+}\)]\(_i\) oscillations (7, 8) based on the well documented biphasic effects of Ca\(^{2+}\) on the IP3-sensitive Ca\(^{2+}\) channel (5, 6). Since these schemes propose that [Ca\(^{2+}\)]\(_i\) oscillations arise directly from the properties of the channels themselves, they have as a corollary that this phe-
nomenon could also occur in permeabilized cells, as confirmed here. In addition, mathematical modeling of these mechanisms, based on experimentally derived parameters for the feed forward and feedback effects of Ca\(^{2+}\) on the IP\(_3\)-sensitive Ca\(^{2+}\) channel, yields theoretical predictions for the oscillatory behavior of [Ca\(^{2+}\)]\(_L\), [Ca\(^{2+}\)]\(_i\), and IP\(_3\)-sensitive Ca\(^{2+}\) channel activity that are remarkably similar to our experimental results (cf. Ref. 8, Fig. 6). In particular, these calculations reproduce the shape of the oscillations in [Ca\(^{2+}\)]\(_L\) described here and, as alluded to above, predict that Ca\(^{2+}\) channels remain open during the initial phase of [Ca\(^{2+}\)]\(_L\) recovery. In these models this delay in channel closure is due to the slow time course of Ca\(^{2+}\) feedback inhibition. The recovery of [Ca\(^{2+}\)]\(_L\), in spite of the fact that the channels remain open, occurs because of the high [Ca\(^{2+}\)]\(_i\) generated during oscillations. This high [Ca\(^{2+}\)]\(_i\) increases Ca\(^{2+}\) influx via the SERCA pump while simultaneously decreasing the driving force for Ca\(^{2+}\) loss via the IP\(_3\)-sensitive channels. The net result is that Ca\(^{2+}\) uptake exceeds loss even before the channels close. In our experiments [Ca\(^{2+}\)]\(_L\) is apparently large enough to duplicate this effect, but because Ca\(^{2+}\) is able to easily diffuse away from the surface of the stores of permeabilized cells, [Ca\(^{2+}\)]\(_L\) recovery is typically incomplete (cf. Figs. 1–3).

Our results provide the first demonstration to date that Ca\(^{2+}\) oscillations can occur in permeabilized cells and suggest that the permeabilized HSY cell preparation described here offers many interesting possibilities for future studies of Ca\(^{2+}\) oscillations.

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