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Cytosolic Ca^{2+} oscillations in REF52 fibroblasts: Ca^{2+}-stimulated IP_3 production or voltage-dependent Ca^{2+} channels as key positive feedback elements

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Abstract —Oscillations in cytosolic free calcium concentrations ($[Ca^{2+}]_i$) can be elicited in REF52 fibroblasts by three different modes of stimulation. We have previously demonstrated that $[Ca^{2+}]_i$ oscillations result when these cells are simultaneously depolarized and stimulated with a hormone linked to phosphoinositide breakdown. Further evidence is now presented that such oscillations are linked to fluctuations in the concentration of IP_3 and the Ca^{2+} content of an IP_3-sensitive Ca^{2+} store. $[Ca^{2+}]_i$ oscillations can also be generated in REF52 cells either by direct stimulation of G-proteins with GTPγS or AlF_4^- or by destabilizing the membrane potential and opening voltage-dependent calcium channels. This report compares the different types of oscillations and their mechanisms.

At least four different models have been proposed to explain calcium oscillations in non-excitable cells (for reviews *see* [1–5]). We have chosen to classify the models according to the following criteria [4, 6]:

(a) *Does PIP_2 breakdown to IP_3 and DG oscillate as well as $[Ca^{2+}]_i$?*
(b) *Does $[Ca^{2+}]_i$ feed back positively or negatively on itself?*

By choosing a positive or negative answer to each of the two criteria, four types of models can be produced. Each is shown as a row of Figure 1 and describes a different mechanism for calcium oscillations already proposed in the literature: **Model 1** – Payne et al. [7] and Parker and Ivorra [8] have noted that high $[Ca^{2+}]_i$ inhibits the ability of IP_3 to release more Ca^{2+} in *Limulus* photoreceptors and *Xenopus* oocytes. This negative feedback could generate oscillations if appropriate kinetic delays are introduced. **Model 2** – Berridge and others have proposed [9–13] that IP_3 releases Ca^{2+} from one intracellular pool, causing Ca^{2+} overload and Ca^{2+}-dependent Ca^{2+} release (CICR) from an IP_3-insensitive store. A mathematical formulation of this model has been developed [14, 15]. **Model 3** – A third model, proposed first by Cobbold and colleagues [10, 16–18], suggests that

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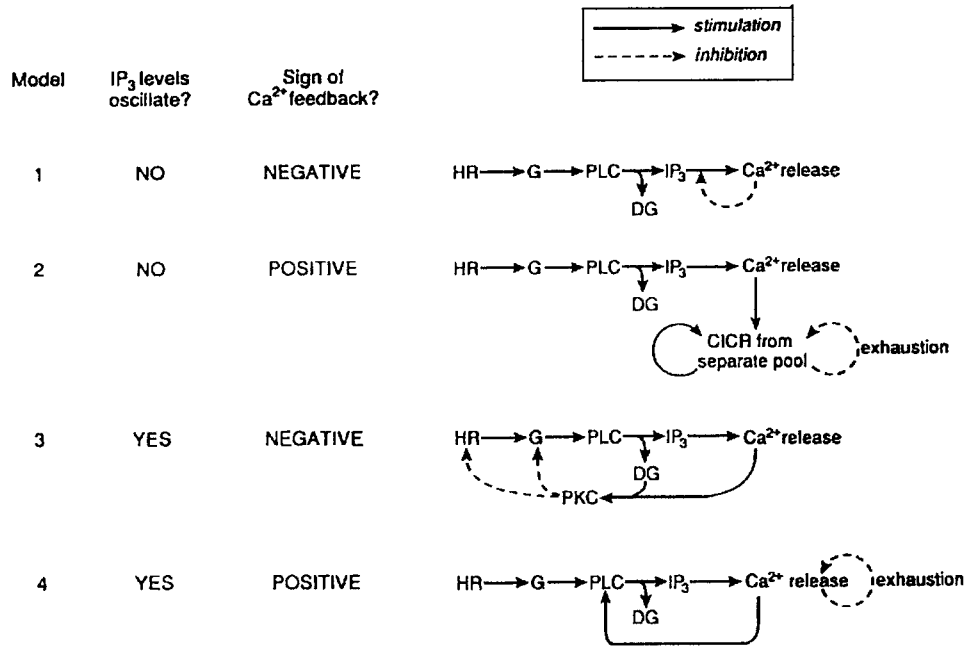


	Model 1	Model 2	Model 3	Model 4
IP_3 releases Ca^{2+} at any phase	–	–	+	+
Caffeine and ryanodine have no effects	+	–	+	+
Ca^{2+} overload without hormone does not start oscillations	+	–	+	+
Ca^{2+} spike (nitr-7) elicits delayed amplification	–	+	–	+
Amplification needs hormone, is blocked by heparin		–		+
IP_3 levels oscillate	–	–	+	+
PKC down regulation does not prevent oscillations			–	
IPS_3 does not start oscillations	–	–	+	+
Thapsigargin stops oscillations	+	–	+	+
Effect of DG pulse lasts several cycles			–	
GTPγS causes oscillations at lower $[Ca^{2+}]_i$	–	–	–	+
GTPγS-induced oscillations are insensitive to PDBu			–	
Ca^{2+} waves spread from local wound	–	+	–	+

Fig. 1 Mechanisms proposed in the literature [1–19] to explain how $[Ca^{2+}]_i$ oscillations may be generated in electrically nonexcitable cells. Abbreviations are as follows: HR, hormone-receptor complex or other activated receptor on the plasma membrane; G, GTP-binding coupling protein; PLC, phospholipase C; DG, diacylglycerol; IP_3, *myo*-inositol-1,4,5-trisphosphate; CICR, Ca^{2+}-induced Ca^{2+} release; PKC, protein kinase C. Stimulatory linkages are shown as solid arrows, inhibitory as dashed arrows. The dashed pathways labeled 'exhaustion' indicate that positive feedback of Ca^{2+} on its own release is soon limited by exhaustion of those stores. The matrix below shows the experimental tests applied, where + and – respectively indicate agreement and disagreement with each theoretical mechanism, and a blank indicates that the theory makes no strong prediction. The first seven rows within the ruled matrix represent tests described in previous papers [6, 20]; the last six rows are the observations reported in the present work

phosphorylations due to protein kinase C are the dominant feedback mechanism controlling the interspike interval. **Model 4** – A fourth model, proposed by Meyer and Stryer [19], is based on positive cooperativity of IP_3 releasing Ca^{2+} from internal stores, together with Ca^{2+} stimulation of phospholipase C to generate additional IP_3.

Cytosolic Ca^{2+} oscillations of unusual metronomic rhythmicity are elicited in REF52 fibroblasts by a combination of (a) stimulation by any of a variety of hormonal agonists and (b) depolarization by any of several pharmacological treatments [20]. Among the effective agonists are serum, vasopressin, thrombin, bradykinin, bombesin, and ATP. What they all have in common is an ability to stimulate phosphoinositide breakdown and IP_3 formation. Effective means of depolarization include high external K^+, the pore-forming antibiotic gramicidin, monovalent cation ionophores nigericin and monensin, sodium-pump inhibition by ouabain, removal of external K^+, or replacement of external Na^+ by Li^+. Either the hormone or depolarization alone elicit a single spike of $[Ca^{2+}]_i$ decaying to a moderately elevated plateau, but applied together they synergistically stimulate repetitive spikes of several hundred nM amplitude and several seconds duration, with an interspike interval in the order of a minute, and continuing for hours. Graded reduction of extracellular Ca^{2+} causes the interspike interval to lengthen with little or no effect on the peak amplitude; this observation suggests that each spike represents release of Ca^{2+} from internal stores, which then need extracellular Ca^{2+} to refill. Blockers of L-type voltage-sensitive Ca^{2+} channels mimic reduction in extracellular Ca^{2+}; this pharmacology suggests that the role of the steady depolarization is to keep L-type channels open and maintain a trickle of Ca^{2+} influx into the cytoplasm. This influx helps replenish the internal stores that actually generate the spikes.

Previous tests [6, 20] aimed at distinguishing between the possible feedback mechanisms in these cells have given results most consistent with the fourth model [19], in which IP_3 levels oscillate because of positive feedback from $[Ca^{2+}]_i$ onto phospholipase C once the latter has already been partially stimulated by G protein coupling. These results are tabulated as the first seven rows of the ruled matrix in Figure 1 and are as follows:

(a) IP_3 can be released suddenly inside the cell by flash photolysis of microinjected caged IP_3. In an otherwise unstimulated cell, this causes a single spike of $[Ca^{2+}]_i$, never oscillations. In cells that are already undergoing oscillations, the IP_3 pulse always generates its own fresh spike of $[Ca^{2+}]_i$ even when the flash coincides with or just follows the falling phase of an endogenous spike. Furthermore, the interval between the exogenously triggered pulse and the next self-generated oscillation is often abnormally long. These results [20] argue that IP_3 release of Ca^{2+} is not sufficient by itself to start oscillations, and favor models in which the Ca^{2+} stores are continually sensitive to IP_3 fluctuations, rather than those in which IP_3 is already statically elevated and the Ca^{2+} stores' sensitivity to IP_3 is what oscillates. The unusually long gap following an exogenously forced, large-amplitude pulse of IP_3 would be most simply explained if the interspike interval is the time required to refill the IP_3-sensitive Ca^{2+} store.

(b) In cell types where calcium-induced Ca^{2+} release (as postulated in Model 2) has been well documented, this process can be stimulated or pre-exhausted by caffeine and ryanodine respectively [21–26]. However, these drugs have no detectable effect on these fibroblasts, either in unstimulated or in oscillating cells [6].

(c) Direct introduction of small or large amounts of Ca^{2+} into the cytoplasm, either by flash photolysis of the photolabile Ca^{2+} chelator nitr-7 [27] or by wounding the cell with a micropipet, never causes oscillation by itself, whereas in Model 2 such a Ca^{2+} overload should mimic dumping of the IP_3-sensitive store in triggering repetitive CICR. However, once hormone is present, wounding then does stimulate oscillation [6].

(d) Detailed examination of the response of an already oscillating cell to flash photolysis of nitr-7 shows that the initial trigger pulse of Ca^{2+} can be temporally separated from a subsequent amplification phase peaking about 10 s later [6]. The delayed amplification shows that Ca^{2+} feedback on itself is positive and could be consistent with either CICR (Model 2) or Model 4. However, the amplification is not observed if hormone is absent. and is blocked in the presence of hormone by

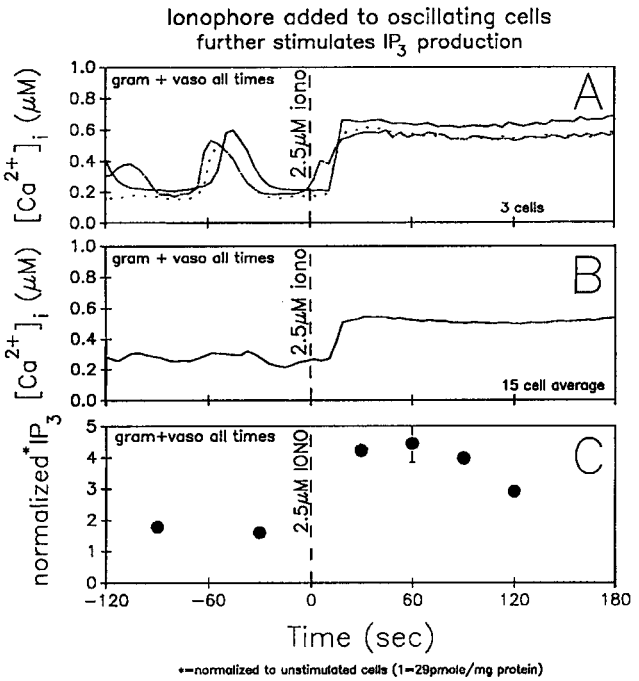


Fig. 2 Ionophore added to oscillating cells further stimulates IP_3 production

(A) 2.5 μM ionomycin halts oscillations and increases $[Ca^{2+}]_i$ in each of 3 cells stimulated to oscillate asynchronously with 50 nM vasopressin and 1 μM gramicidin in a solution of Dulbecco's phosphate buffered saline (DPBS) containing 0.9 mM Ca^{2+} and 5.6 mM glucose. $[Ca^{2+}]_i$ increases to a level approximately equal to that seen at the peak of the oscillatory spikes in each cell

(B) Average $[Ca^{2+}]_i$ for 15 cells including the 3 cells plotted in (A)

(C) IP_3 levels measured using an IP_3 mass assay as described previously [6]. Note that both IP_3 mass and average $[Ca^{2+}]_i$ approximately double with roughly the same time course. All measurements were performed at 30°C

intracellular heparin, an antagonist of the IP_3 receptor [28–30]. It is most readily explained by Ca^{2+} stimulation of IP_3 production leading to release of more Ca^{2+}.

(e) Direct measurements of IP_3 can still only be done on populations rather than single cells, so to test whether IP_3 concentrations oscillate it is necessary to synchronize the population. This can be roughly accomplished by a protocol of withdrawing then restoring extracellular Ca^{2+}. The first spike of the resuming oscillations occurs fairly synchronously about 30 s after external Ca^{2+} is added back; parallel measurements indicate a similar spike in IP_3 at this time. Afterwards the individual oscillations fall out of synchrony, so that the average $[Ca^{2+}]_i$ decays to an intermediate value; the IP_3 assayed in the population does the same [6]. Further confirmation of the ability of cytosolic Ca^{2+} to influence IP_3 levels comes from experiments with the Ca^{2+} ionophore ionomycin. When ionomycin is used to elevate $[Ca^{2+}]_i$ in otherwise unstimulated cells to the level found at the peak of normal oscillations, IP_3 levels do not change significantly. Stimulation of oscillations with hormone and depolarization, without ionomycin, gives a modest (1.8 fold) elevation of IP_3 above control. Addition of ionomycin to oscillating cells clamps the $[Ca^{2+}]_i$ to its previous peak levels and gives IP_3 levels 4.5 fold over unstimulated controls (Fig. 2). Thus $[Ca^{2+}]_i$ elevation further stimulates phospholipase C but only after the latter has been initially activated by hormone receptor occupancy coupled through G proteins. Phospholipase C activity has usually been found to be enhanced by increased Ca^{2+} [31–39], though contrary reports exist [40].

(f) Though oscillations are very easily inhibited by acute addition of low doses of phorbol ester to activate protein kinase C, such sensitivity does not prove that cyclical activation of protein kinase C is an essential part of the oscillation mechanism. When PKC is inhibited pharmacologically or thoroughly removed by downregulation, oscillations can still be elicited in the normal way by hormone plus depolarization [6], showing that PKC is nonessential and arguing strongly against Model 3.

We now present additional pharmacological results in favor of Model 4 and show how two additional modes by which oscillations may be stimulated can be fitted in.

Materials and Methods

General methods for culturing the REF52 cells, for stimulating them and measuring $[Ca^{2+}]_i$ oscillations in single cells by digital ratio imaging, and for photolyzing caged compounds have been described previously [6, 20, 41]. Fura-2 was used as the Ca^{2+} indicator in most experiments because $[Ca^{2+}]_i$

concentrations could be quantified via the excitation ratio. Fluo–3 was used for experiments involving photolysis of caged compounds, since the UV excitation required for Fura–2 would start the photolyses prematurely. Intracellular pressure microinjections are described in terms of the solutions filling the pipet; because the injected volume was 1–10% of the cell volume, the final intracellular concentrations are roughly 1–10% of the filling solution. Details of specific experiments are given in the appropriate figure legends.

Caged *sn*-1,2-dioctanoylglycerol, 3-(4,5-dimethoxy-2-nitrobenzyl)-*sn*-1,2-dioctanoylglycerol, was prepared as follows: (4,5-dimethoxy-2-nitrophenyl) diazomethane was prepared from 4,5-dimethoxy-2-nitrobenzaldehyde mesitylene-sulfonylhydrazone by the method of Dudman & Reese [42]. The diazomethane (18 mg or 80 μmol) and 25 mg (72.5 μmol) of *sn*-1,2-dioctanoylglycerol (diC_8, Calbiochem Corp., La Jolla, CA, USA) were mixed in 0.2 ml $CHCl_3$. Upon addition of $BF_3{\bullet}Et_2O$ (7 μmol as 1 M solution in $CHCl_3$), nitrogen gas was rapidly evolved. Thin layer chromatography (on silica with ethyl acetate-hexane as eluant) indicated complete reaction yielding one major product, purified by column chromatography with the same system. The product was isolated as a yellow oil, which eventually solidified to a white powder on storage at –20°C — m.p. 39°C; yield 15 mg (38%). It gave the expected ^1H NMR spectrum ($CDCl_3$, 200MHz): δ 0.85 ppm (broad triplet, 6H, CH_3), 1.26 (broad multiplet, 16H, $(CH_2)_4$), 1.61 (multiplet, 4H, $-COCH_2CH_2-$), 2.32 (triplet, 2H, $-COCH_2-$), 2.33 (triplet, 2H, $-COCH_2-$), 3.76 (doublet, J = 5.4 Hz, 2H, CH_2 of glycerol position 3), 3.96 (singlet, 3H, $-OCH_3$), 4.01 (singlet, 3H, $-OCH_3$), 4.22 (doublet of doublets, 1H, CH_2 at glycerol position 1), 4.39 (doublet of doublets, 1H, CH_2 at glycerol position 1), 4.94 (singlet, 2H, $ArCH_2-$), 5.36 (m, 1H, CH at glycerol position 2), 7.26 (singlet, 1H, H at aryl position 3), 7.72 (singlet, 1H, H at aryl position 6). It was stored at –20°C until use; stock solutions were made up in DMSO. Its extinction coefficient at 365 nm was 5500 $M^{-1}cm^{-1}$. Its photolysis quantum yield at that wavelength, monitored by actinometry [27] and HPLC loss of starting material, was 0.09 in CH_3CN:H_2O (7:3 v/v) containing 10 mM K^+-MOPS pH 7.2.

Results and Discussion

Effects of agents to release the IP_3-sensitive Ca^{2+} store

A major argument that $[Ca^{2+}]_i$ oscillations in other cell types do not involve IP_3 oscillations has been that direct introduction of IP_3, or better yet its poorly hydrolyzable analog *myo*-inositol-1,4,5-trisphosphorothioate [43, 44] (herein abbreviated as IPS_3), is sufficient to generate apparently normal $[Ca^{2+}]_i$ oscillations [12, 45, 46]. As mentioned previously, IP_3 by itself does not cause oscillations in REF52 fibroblasts, but it was still of interest to try IPS_3. Injections of IPS_3 at pipet concentrations of up to 20 mM were therefore made. In all cases a transient $[Ca^{2+}]_i$ increase followed by a smooth non-oscillatory return to baseline was observed (Fig. 3A). Some but not all of the $[Ca^{2+}]_i$ transient could have been due to impalement damage, since when injections were performed in the absence of extracellular Ca^{2+}, $[Ca^{2+}]_i$ transients were still observed, albeit smaller in amplitude (Fig. 3B). These results would suggest that non-oscillatory activation of the IP_3 receptor is insufficient to generate $[Ca^{2+}]_i$ oscillations in these cells. One qualification is that we have no proof of how long the IPS_3 persists after each injection; the successively diminishing responses to repeated injections in Figure 3B hints that some effectiveness is persisting.

The effect of the tumor promoter thapsigargin on both oscillating and non-oscillating cells was examined. Thapsigargin has been reported to inhibit the endoplasmic reticulum ATP-dependent Ca^{2+} pump and to release Ca^{2+} from the associated store, with little or no effect upon plasma membrane and sarcoplasmic reticulum Ca^{2+} pumps [47–49]. When REF52 cells were exposed to 2 μM thapsigargin, $[Ca^{2+}]_i$ increased from 85 nM to a stable value of 250 nM, but no $[Ca^{2+}]_i$ oscillations were observed (Fig. 4A), even if the cells were simultaneously depolarized with 1 μM gramicidin. However, oscillations started in the normal way were quenched by thapsigargin (Fig. 4B). Thus the intracellular Ca^{2+} store which is emptied and refilled during the course of $[Ca^{2+}]_i$ oscillations in REF52 fibroblasts appears to be sensitive to thapsigargin.

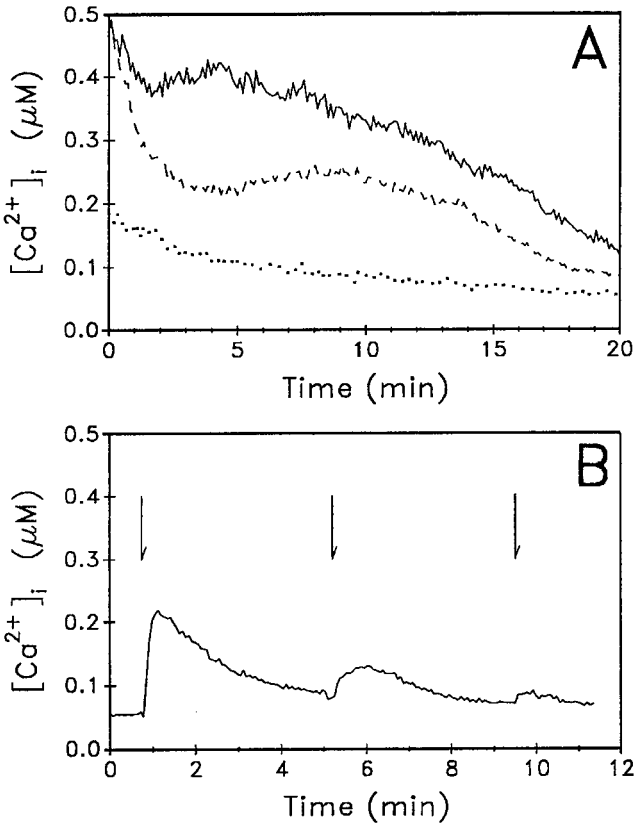


Fig. 3 Responses to inositol 1,4,5-trisphosphorothioate

(**A**) Records of $[Ca^{2+}]_i$ in three cells just after injection with a solution containing 10 mM IPS_3 (triethylammonium salt, racemic), 500 μM Fura–2 (K^+ salt), and 20 mM K-HEPES. Note the absence of $[Ca^{2+}]_i$ oscillations (compare with Fig. 7A). The extracellular medium was DPBS containing 0.9 mM Ca^{2+} and 5.6 mM glucose; temperature 30°C. The actual injections occurred 1–3 min before the time zero of this plot; the time delay resulted from having to inject several cells, then to place the measurement spots. However no oscillations were noticed in the $[Ca^{2+}]_i$ images taken between the injections and the start of the quantification. Pipet concentrations in other runs were 0.1, 0.3, 1, 3, 10, and 20 mM, but oscillations were never observed

(**B**) Arrows indicate 3 successive injections of a single cell with a solution of 5 mM IPS_3, 20 mM K-HEPES, and 1 mM EGTA to chelate any contaminating Ca^{2+} in the nominally Ca^{2+}-free injectate. Pressure injections were performed in calcium-free DPBS containing 100 μM EGTA, 10 mM Mg^{2+} and 5.6 mM glucose; temperature 30°C. The Fura–2 was preloaded via the acetoxymethyl ester. The diminished response with successive injections was not due to accumulation of intracellular EGTA, because control experiments in which 1 mM IP_3, 1 mM EGTA was injected showed that at least six separate releases of Ca^{2+} could be elicited under the same conditions

This result is in sharp contrast to those recently reported in pancreatic acinar cells [26]. In these cells thapsigargin causes oscillations, apparently by specifically depleting the IP_3-sensitive Ca^{2+} pool, which then provokes cyclical CICR from another pool that is insensitive to thapsigargin and IP_3 but sensitive to caffeine and ryanodine.

Effects of diacylglycerol

Though phorbol esters have profound acute inhibitory effects on the Ca^{2+} oscillations, a diacylglycerol such as diC_8, *sn*-1,2-dioctanoylglycerol [50], would be a more realistic analog of the endogenous activators of protein kinase C. DiC_8 at 2–4 μM concentrations in the bath slightly increased the interspike interval (by up to a factor of 2 at most) with variable effects on spike amplitude. Higher concentrations of diC_8 did not increase the effect. In order to examine the kinetics of the effect in greater detail, it was desirable to deliver a pulse of diC_8 at a well-defined time in the oscillation cycle. Because of the uncertain time required for bath-applied diC_8 to cross the plasma membrane and bind to intracellular protein kinase C, it would be useful to have caged diC_8, a physiologically inert derivative which could be photolyzed *in situ* to release the active material with minimal diffusional delays. The caging moiety was chosen to be the dimethoxynitrobenzyl group [51] because of its efficient photolysis with relatively long-wavelength ultraviolet. The caged diC_8 behaved as desired, with no effect on the oscillations until photolyzed, at which point the interspike interval increased. In the experiment of Figure 5, the first interval (averaged over 16 cells) after the flash was about 30% longer than before the photolysis. Interestingly, the effect lasted for several oscillation cycles before decaying away. If the interspike timing mechanism were metabolism of diacylglycerol and reversal of protein kinase C-mediated phosphorylations as in Model 3, one would expect that an exogenous pulse of diacylglycerol would give just one abnormally long waiting period until the excess was consumed and the next Ca^{2+} spike was allowed to occur, after which no memory would persist. The actual result suggests that PKC modulates the oscillations over a slower time scale than postulated in Model 3;

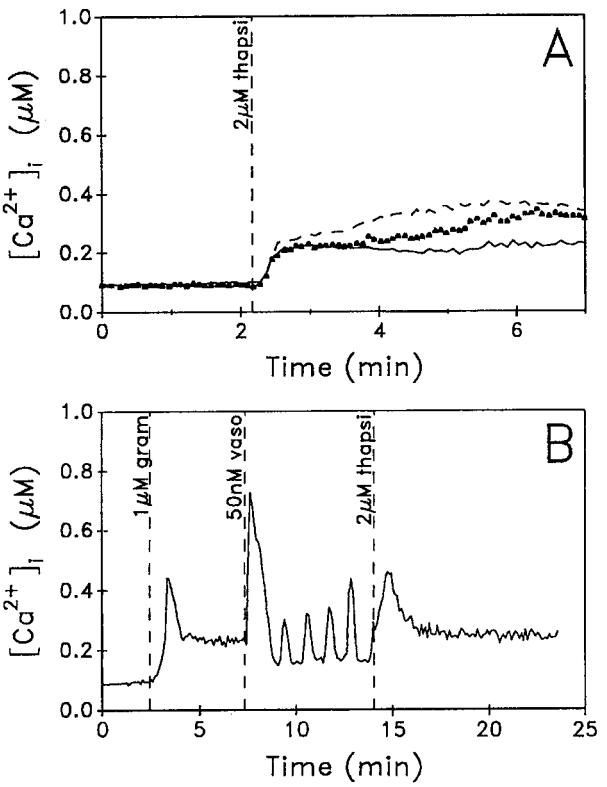


Fig. 4 Responses to thapsigargin. (**A**) In previously unstimulated cells, 2 μM thapsigargin causes a monotonic increase of $[Ca^{2+}]_i$ by more than two-fold in 3 cells. Temperature was 30°C. No oscillations were observed in any of 45 REF52 cells treated with thapsigargin. (**B**) Thapsigargin (2 μM) halts $[Ca^{2+}]_i$ oscillations initiated by 1 μM gramicidin and 50 nM vasopressin. In this experiment thapsigargin halted oscillations in 5 of 5 oscillating cells. Temperature was 33°C. Thapsigargin was obtained from LC Services Corp., Woburn, MA, USA

however, it must be acknowledged that the diC_8 is still not the same as endogenous diacylglycerol either in structure or in intracellular location.

Calcium oscillations are elicited by direct activation of G-proteins

Direct activation of G-proteins elicits calcium oscillations in several different tissue types including golden hamster eggs [52], pancreatic acinar cells [13], hepatocytes [53, 54], and smooth muscle-like cells (AT Harootunian, unpublished results). We found that injection with pipets containing 1–18 mM GTPγS reproducibly produced oscillations in $[Ca^{2+}]_i$. Oscillations could also be elicited by photolyzing caged-GTPγS which had been preinjected into cells or by treating cells with AlF_4^- (10 mM NaF and 10 μM $AlCl_3$). The convenience of extracellularly applied AlF_4^- was offset by its tendency to cause gradual deterioration of the cells, presumably by nonspecific toxicity. These oscillations did not require additional depolarization of the membrane potential. Furthermore they were not inhibited by blockers of voltage-dependent Ca^{2+} channels, removal of extracellular Ca^{2+} (Fig. 6A), or even high doses of phorbol esters (Fig. 7A), unlike hormone-stimulated oscillations (Figs. 6B and 7B). However, many responses of the timing mechanism did appear to be shared between the two modes of stimulation:

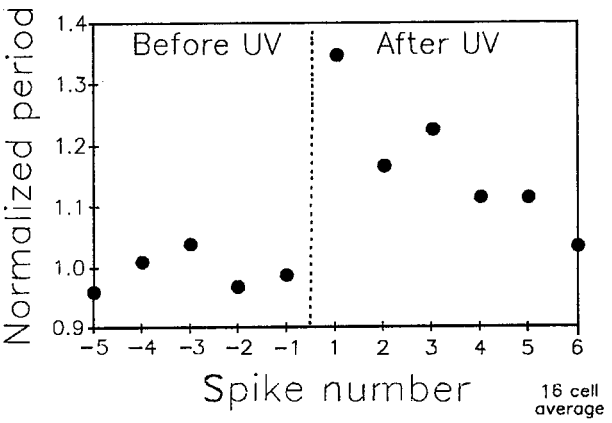


Fig. 5 Photolysis of caged diacylglycerol increases $[Ca^{2+}]_i$ oscillation period for several cycles. The normalized oscillation period, averaged over 16 cells from three separate experiments, increased by a factor of 1.3 following photolysis of caged diacylglycerol, which was loaded into the cells by continuous incubation at 20 μM (two experiments) or 40 μM (one experiment) in HEPES-buffered saline. Photolysis was performed as described previously [20] with a 1 s exposure to broadband UV illumination from a 150-watt xenon lamp introduced through the epi-illumination system, and is indicated by the vertical dashed line. The average oscillation period for the 16 cells before photolysis was 40 ± 9 s. Temperature was 33°C. To control for the effects of illumination and the 4,5-dimethoxy-2-nitrosobenzaldehyde byproduct of the photolytic reaction, analogous experiments were performed with caged methanol, i.e. 4,5-dimethoxy-2-nitrobenzyl methyl ether. Photolysis of this material (at similar concentrations to caged diC_8) to produce the above benzaldehyde plus methanol had no effect on the oscillations

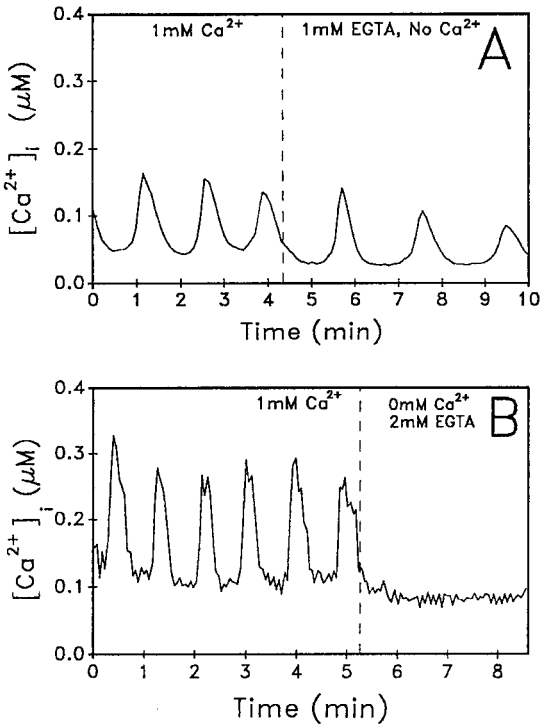


Fig. 6 (A) Ca^{2+} oscillations elicited by G-protein hyperactivation persist after removal of extracellular $[Ca^{2+}]_i$. G-proteins were activated by injection with a solution containing 15 mM GTPγS, 500 μM Fura–2 and 25 mM K^+-HEPES. Temperature 27°C
(B) Conversely, removal of extracellular $[Ca^{2+}]_i$ rapidly halts oscillations elicited by hormone and depolarization (50 nM vasopressin and 1 μM gramicidin). Temperature 29°C

photorelease of caged IP_3 released Ca^{2+} at any phase of the cycle and was followed by one abnormally long interspike interval (Fig. 8); photorelease of caged Ca^{2+} from nitr-7 reset the oscillation phase; thapsigargin stopped the oscillations. One key observation was that GTPγS-induced oscillations usually occurred at a lower absolute $[Ca^{2+}]_i$ than those elicited by hormone plus depolarization (Fig. 9; also compare Figs 6A and 6B with 7A and 7B).

These seemingly paradoxical results fit nicely into Model 4 if one postulates that hyperactivation of the G protein shifts the Ca^{2+}-dependence of phospholipase C down to resting or lower $[Ca^{2+}]_i$ concentrations. Just such a shift has been reported in several studies of G protein effects on the phospholipase in vitro [36–39]. One must also postulate that reuptake of Ca^{2+} into the IP_3-sensitive stores still occurs at these lower $[Ca^{2+}]_i$ levels, whereas extrusion across the plasma membrane is disfavored. Continual entry of extracellular Ca^{2+} through the voltage-dependent channels would no longer be needed to keep $[Ca^{2+}]_i$ in the region where it could exert positive feedback, but otherwise the mechanism would be unchanged. The lack of effectiveness of protein kinase C feedback emphasizes that PKC is not essential for oscillations and argues that its modulatory effect is normally exerted upstream from the activated G protein, for example at the hormone receptor or its promotion of the GTP-bound state of the G protein, rather than downstream, for example at phospholipase C. This conclusion in REF52 cells may differ from indications in hepatocytes [53] that phorbol esters may inhibit G proteins or PLC there. With any of the alternative oscillation mechanisms, one would

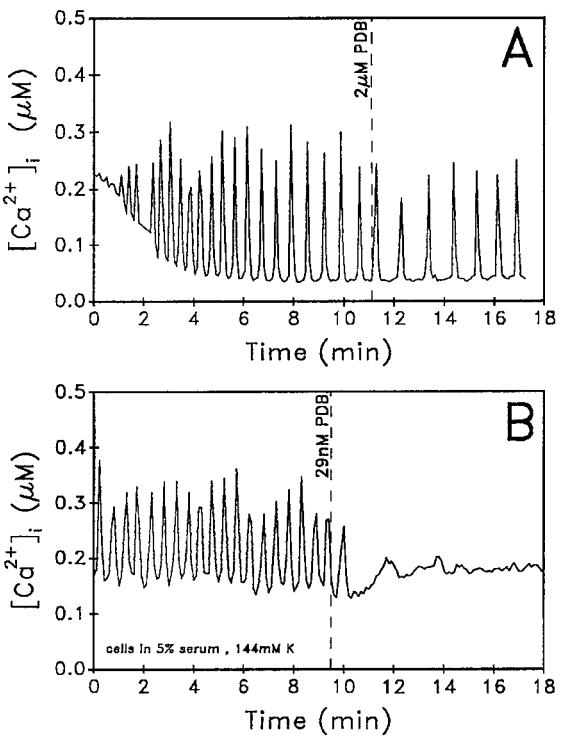


Fig. 7 Protein kinase C activation differentially affects oscillations. (A) Treatment with even a high level, 2 μM, of phorbol dibutyrate (PDB) only transiently slows oscillations elicited by G-protein activation in a manner analogous to Figure 6A. Temperature was 25°C. (B) A much lower dose of PDB, 29 nM, immediately halts oscillations elicited by mitogen (5% fetal calf serum) and depolarization (144 mM K^+); temperature 30°C

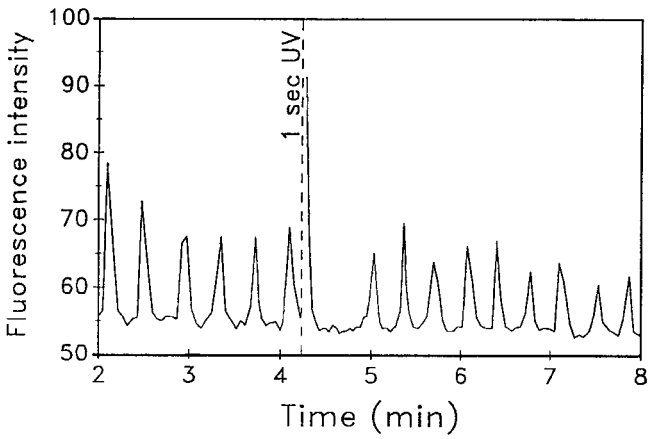
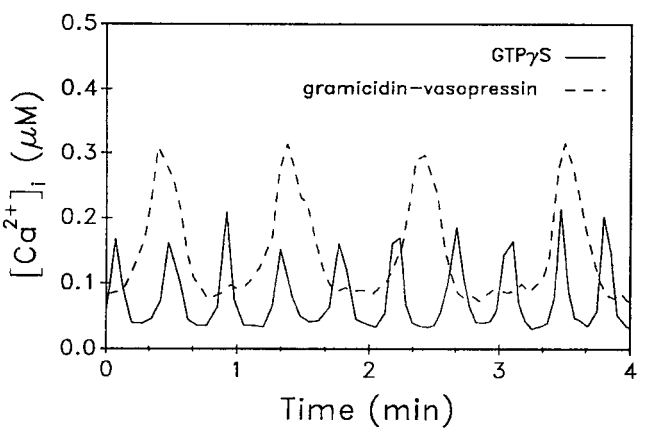


Fig. 8 After photolysis of caged IP_3, the time interval until the next endogenous spike can be more than twice the normal oscillation period. Oscillations were elicited by G-protein hyperactivation by injection of a solution containing 11 mM GTPγS, 10 mM Fluo-3, 1 mM caged IP_3 and 20 mM K^+-HEPES. Temperature 30°C. Photolysis was performed with 1 s ultraviolet as in Figure 5; this exposure should have been sufficient to photolyze most of the caged IP_3. To control for the effects of illumination as well as the 2-nitrosoacetophenone and H^+ also released by the photolytic reaction, analogous experiments were performed with caged phosphate, i.e. 1-(2-nitrophenyl)ethyl-phosphate. When this material was injected analogously to caged IP_3 and photolyzed to 2-nitrosoacetophenone and phosphoric acid, the oscillations were unaffected

have to postulate that G protein hyperactivation shifts the Ca^{2+}-dependence of inhibition of IP_3-mediated release, activation of CICR, or PKC activation, though there is no evidence that any of these processes are directly linked to G proteins.

One further aspect of oscillations due to GTPγS is that sometimes they can be clearly seen as repetitive waves, which originate from a region of slightly elevated $[Ca^{2+}]_i$ where the injection pipet had wounded the cell. As the wound heals, the $[Ca^{2+}]_i$ elevation becomes more nearly synchronous throughout the cell. These observations support the idea that Ca^{2+} feedback exerts positive rather than negative feedback on itself. The details of such waves and whether they also underlie the responses to hormone and depolarization have not yet been studied, partly because the time resolution of the imaging setup would need considerable improvement.



Synchronized $[Ca^{2+}]_i$ oscillations can be generated by destabilizing membrane potential and opening L-type Ca^{2+} channels

The existence of L-type voltage-sensitive Ca^{2+} channels in fibroblasts is supported both by our pharmacological results and by the patch-clamp analysis of Chen & Hess in 3T3 cells [55]. It would be interesting if those channels could give rise to voltage-linked $[Ca^{2+}]_i$ spiking as classically observed in excitable cells. To encourage voltage fluctuations, 1 mM 4-aminopyridine and 10 mM tetraethylammonium were added to block K^+ channels and 5–20 μM BayK 8644 added to encourage the opening of L-type Ca^{2+} channels. This third mode of stimulation did produce $[Ca^{2+}]_i$ oscillations, as shown in Figure 10. A remarkable feature of channel-driven oscillations was that in confluent cultures all the cells in the field (typically 15–20 cells) rhythmically oscillated in unison (Fig.

Fig. 9 $[Ca^{2+}]_i$ is lower in cells stimulated to oscillate by G-protein activation (solid trace) than in cells in which oscillations are elicited by hormone and depolarization (dashed line). Temperature 34°C. Although the maximum $[Ca^{2+}]_i$ levels in oscillating cells were somewhat variable from cell to cell, the baseline $[Ca^{2+}]_i$ for oscillations elicited by G-protein was typically 50 nM while the baseline $[Ca^{2+}]_i$ for oscillations elicited by hormone and depolarization was usually near or greater than 100 nM. Because neither form of stimulation was easily reversible, this comparison had to be made on closely parallel experiments rather than sequentially on the same cells. Although the absolute overall numerical calibration of the $[Ca^{2+}]_i$ measurements is probably good only within a factor of two, the relative $[Ca^{2+}]_i$ levels produced by the two modes of stimulation can still be assessed with reasonable confidence

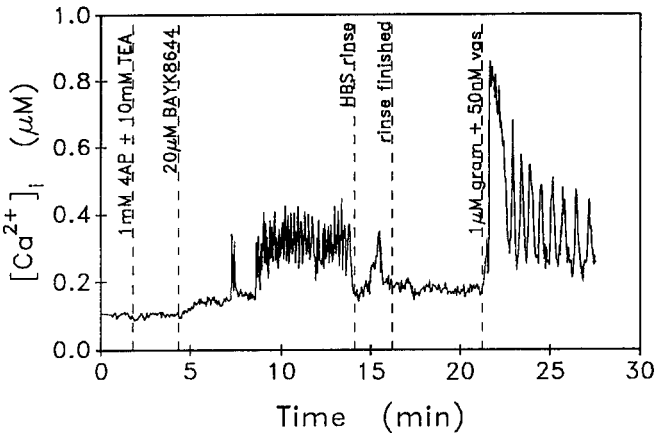


Fig. 10 Channel-driven $[Ca^{2+}]_i$ oscillations elicited by 1 mM 4-aminopyridine (4AP), 10 mM tetraethylammonium (TEA), and 20 μM BayK 8644. After these drugs were washed away, the cell could be restimulated into oscillations with 1 μM gramicidin and 50 nM vasopressin. Temperature was 35°C

11A), whereas in oscillations induced by the previous two modes of stimulation, each cell was independent of its neighbor (Fig. 11B). Another difference was that channel-driven oscillations were halted by static depolarization of the cells either with high potassium or gramicidin. The IP_3-sensitive internal store remained replete with Ca^{2+} throughout channel-driven oscillations, since addition of a hormone such as vasopressin to the channel modulators always gave a large spike of $[Ca^{2+}]_i$ (experiments not shown). Subsequent behavior in the combined presence of hormone and channel modulators was variable from cell to cell.

A tentative interpretation of these results is that the encouragement of L-type Ca^{2+} channels and blockage of resting K^+ channels would permit spontaneous slow Ca^{2+} 'action potentials', in which the upstroke would be driven by regenerative opening of the Ca^{2+} channels, and repolarization might be caused by a Ca^{2+}-activated K^+ conductance. Synchronization of the entire field of cells would be via electrotonic coupling. By contrast, oscillations caused by hormone plus static depolarization would not be synchronized, since electrotonic coupling would be irrelevant when all the cells were already clamped to the same depolarized potential, and a chemical messenger such as IP_3 would spread much less efficiently than a membrane potential change. To confirm this interpretation, it would be desirable, though difficult, to conduct electrical recordings from the cells undergoing channel-driven oscillations and to use localized illumination of caged IP_3 to show the extent to which IP_3 action can spread from cell to cell. Though IP_3 is believed to be able to cross gap junctions in liver [56] and epithelial [57] cells, the REF52 fibroblasts may not pack together as closely, so the few contacts they make at the tips of their processes may be insufficient for effective chemical coupling.

Conclusion

REF52 fibroblasts have been an interesting system for studying $[Ca^{2+}]_i$ oscillations because they are relatively easy to microinject, because their

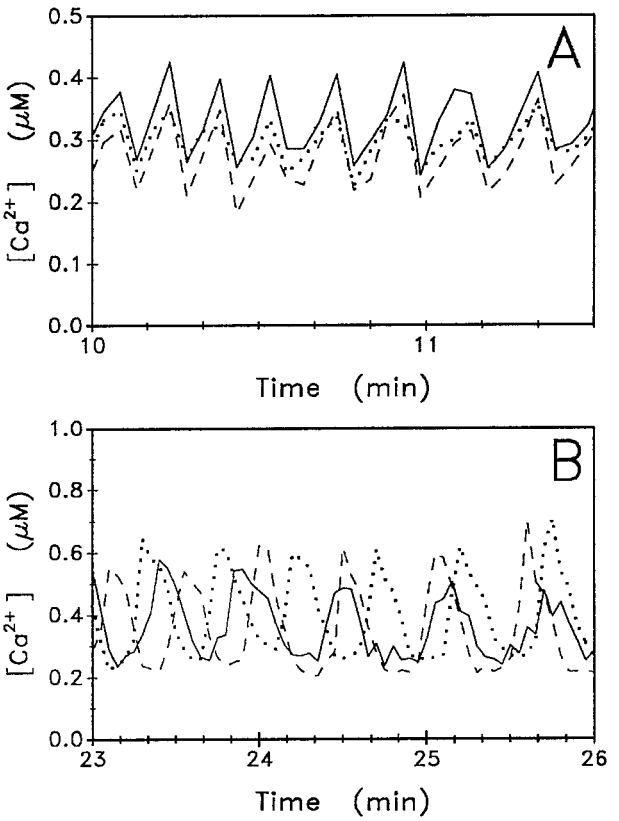


Fig. 11 Expanded $[Ca^{2+}]_i$ traces from three cells from the same experiment as Figure 10 show that the oscillations elicited by 4AP, TEA, and BayK 8644 (**A**) were synchronized within the population, whereas the subsequent oscillations elicited by vasopressin and gramicidin (**B**) were asynchronized

oscillations are unusually accurate in timing, thus permitting phase analysis, and because they have given many lines of evidence (Fig. 1) in favor of a fascinating oscillation mechanism (Model 4 [19]) other than the currently most popular explanation (Model 2), based on Ca^{2+}-induced Ca^{2+} release. The comparison between the three modes of stimulation is particularly instructive. However, the REF52 system does have drawbacks. The hormonal agonists are of high affinity and hard to wash off, so that dose-response curves and investigations of store refilling are difficult. The need for pharmacological depolarization to accompany hormonal stimulation casts doubt on the physiological role of oscillations, and there is no obvious cell function that can be monitored as an immediate response to $[Ca^{2+}]_i$ elevations. Our eventual understanding of the mechanisms and functions of $[Ca^{2+}]_i$ oscillations will have to be based on comparison of a wide variety of systems, without the prejudice that they must all use a single mechanism or have a common purpose.

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References

1. Berridge MJ. (1990) Calcium oscillations. J. Biol. Chem., 265, 9583-9586.
2. Jacob R. (1990) Calcium oscillations in electrically non-excitable cells. Biochim. Biophys. Acta, 1052, 427-438.
3. Rink TJ. Merritt JE. (1990) Calcium signalling. Curr. Opinion Cell Biol., 2, 198-205.
4. Tsien RW. Tsien RY. (1990) Calcium channels, stores, and oscillations. Annu. Rev. Cell Biol., 6, 715-760.
5. Meyer T. Stryer L. (1991) Calcium spiking. Annu. Rev. Biophys. Biophysical Chem., 20, 153-174.
6. Harootunian AT. Kao JPY. Paranjape S. Tsien RY. (1991) $[Ca^{2+}]_i$ oscillations in REF52 fibroblasts are produced by positive feedback between $[Ca^{2+}]_i$ and IP_3. Science, 251, 75-78.
7. Payne R. Flores TM. Fein A. (1990) Feedback inhibition by calcium limits the release of calcium by inositol trisphosphate in *Limulus* ventral photoreceptors. Neuron, 4, 547-555.
8. Parker I. Ivorra I. (1990) Inhibition by Ca^{2+} of inositol trisphosphate-mediated Ca^{2+} liberation: a possible mechanism for oscillatory release of Ca^{2+}. Proc. Natl. Acad. Sci. USA, 87, 260-264.
9. Berridge MJ. Irvine RF. (1989) Inositol phosphates and cell signalling. Nature, 341, 197-205.
10. Berridge MJ. Cobbold PH. Cuthbertson KSR. (1988) Spatial and temporal aspects of cell signalling. Philos. Trans. R. Soc. Lond. [Biol], 320, 325-343.
11. Rooney TA. Sass EJ. Thomas AP. (1989) Characterization of cytosolic calcium oscillations induced by phenylephrine and vasopressin in single fura-2-loaded hepatocytes. J. Biol. Chem., 264, 17131-17141.
12. Wakui M. Potter BVL. Petersen OH. (1989) Pulsatile intracellular calcium release does not depend on fluctuations in inositol trisphosphate concentration. Nature, 339, 317-320.
13. Osipchuk YV. Wakui M. Yule DI. Gallacher DV. Petersen OH. (1990) Cytoplasmic Ca^{2+} oscillations evoked by receptor stimulation, G-protein activation, internal application of inositol trisphosphate or Ca^{2+}: simultaneous microfluorimetry and Ca^{2+}-dependent Cl^- current recording in single pancreatic acinar cells. EMBO J. 9, 697-704.
14. Goldbeter A. Dupont G. Berridge MJ. (1990) Minimal model for signal-induced Ca^{2+} oscillations and for their frequency encoding through protein phosphorylation. Proc. Natl. Acad. Sci. USA, 87, 1461-1465.
15. Dupont G. Berridge MJ. Goldbeter A. (1990) Latency correlates with period in a model for signal-induced Ca^{2+} oscillations based on Ca^{2+}-induced Ca^{2+} release. Cell Regul., 1, 853-861.
16. Woods NM. Cuthbertson KSR. Cobbold PH. (1987) Agonist-induced oscillations in cytoplasmic free calcium concentration in single rat hepatocytes. Cell Calcium, 8, 79-100.
17. Sanchez-Bueno A. Dixon CJ. Woods NM. Cuthbertson KSR. Cobbold PH. (1990) Inhibitors of protein kinase C prolong the falling phase of each free-Ca transient in a hormone-stimulated hepatocyte. Biochem. J., 268, 627-32.
18. Sage SO. Adams DJ. van Breemen C. (1989) Synchronized oscillations in cytoplasmic free calcium concentration in confluent bradykinin-stimulated bovine pulmonary artery endothelial cell monolayers. J. Biol. Chem., 264, 6-9.
19. Meyer T. Stryer L. (1988) Molecular model for receptor-stimulated calcium spiking. Proc. Natl. Acad. Sci. USA, 85, 5051-5055.
20. Harootunian AT. Kao JPY. Tsien RY. (1988) Agonist-induced calcium oscillations in depolarized fibroblasts and their manipulation by photoreleased Ins(1,4,5)P_3, Ca^{2+}, and Ca^{2+} buffer. Cold Spring Harbor Symp. Quant. Biol., 53, 945-953.
21. Fabiato, A. (1983) Calcium-induced release of calcium from the cardiac sarcoplasmic reticulum. Am. J. Physiol. 245, C1-C14.
22. Valdeolmillos M. O'Neill SC. Smith GL. Eisner DA. (1989) Calcium-induced calcium release activates contraction in intact cardiac cells. Pflügers Arch., 413, 676-678.
23. Lipscombe D. Madison DV. Poenie M. Reuter H. Tsien RW. Tsien RY. (1988) Imaging of cytosolic Ca^{2+} transients arising from Ca^{2+} stores and Ca^{2+} channels in sympathetic neurons. Neuron, 1, 355-365.
24. Kuba K. (1980) Release of calcium ions linked to the activation of potassium conductance in a caffeine-treated sympathetic neurone. J. Physiol., 298, 251-269.
25. Malgaroli A. Fesce R. Meldolesi J. (1990) Spontaneous $[Ca^{2+}]_i$ fluctuations in rat chromaffin cells do not require inositol 1,4,5-trisphosphate elevations but are generated by a caffeine and ryanodine-sensitive intracellular Ca^{2+} store. J. Biol. Chem., 265, 3005-3008.

26. Foskett JK. Wong D. (1990) Caffeine- and ryanodine-sensitive Ca oscillations in thapsigargin-treated acinar cells. J. Cell Biol., 111, 466a.
27. Adams SR. Kao JPY. Grynkiewicz G. Minta A. Tsien RY. (1988) Biologically useful chelators that release Ca^{2+} upon illumination. J. Am. Chem. Soc., 110, 3212-3220.
28. Kobayashi S. Kitazawa T. Somlyo AV. Somlyo AP. (1989) Cytosolic heparin inhibits muscarinic and alpha-adrenergic Ca^{2+} release in smooth muscle. Physiological role of inositol 1,4,5-trisphosphate in pharmacomechanical coupling. J. Biol. Chem., 264, 17997-18004.
29. Palade P. Dettbarn C. Alderson B. Volpe P. (1989) Pharmacologic differentiation between inositol-1,4,5-trisphosphate-induced Ca^{2+} release and Ca^{2+}- or caffeine-induced Ca^{2+} release from intracellular membrane systems. Mol. Pharmacol., 36, 673-680.
30. Ghosh TK. Eis PS. Mullaney JM. Ebert CL. Gill DL. (1988) Competitive, reversible, and potent antagonism of inositol 1,4,5-trisphosphate-activated calcium release by heparin. J. Biol. Chem., 263, 11075-11079.
31. Eberhard DA. Holz RW. (1988) Intracellular Ca2+ activates phospholipase C. Trends Neurosci., 11, 517-520.
32. Rhee SG. Suh PG. Ryu SH. Lee SY. (1989) Studies of inositol phospholipid-specific phospholipase C. Science, 244, 546-550.
33. McDonough PM. Goldstein D. Brown JH. (1988) Elevation of cytoplasmic calcium concentration stimulates hydrolysis of phosphatidylinositol bisphosphate in chick heart cells: effect of sodium channel activators. Mol. Pharmacol., 33, 310-315.
34. Chandler LJ. Crews FT. (1990) Calcium- versus G protein-mediated phosphoinositide. Hydrolysis in rat cerebral cortical synaptoneurosomes. J. Neurochem., 55, 1022-1030.
35. Morris AJ. Waldo GL. Downes CP. Harden TK. (1990) A receptor and G-protein-regulated polyphosphoinositide-specific phospholipase C from turkey erythrocytes. I. Purification and properties. J. Biol. Chem., 265, 13501-13507.
36. Morris AJ. Waldo GL. Downes CP. Harden TK. (1990) A receptor and G-protein-regulated polyphosphoinositide-specific phospholipase C from turkey erythrocytes. II. P_{2Y}-purinergic receptor and G-protein-mediated regulation of the purified enzyme reconstituted with turkey erythrocyte ghosts. J. Biol. Chem., 265, 13508-13514.
37. Taylor SJ. Exton JH. (1987) Guanine-nucleotide and hormone regulation of polyphosphoinositide phospholipase C activity of rat liver plasma membranes. Biochem. J., 248, 791-799.
38. Guillon G. Mouillac B. Berrada R. Balestre MN. Lombard C. (1989) Properties of membranous phospholipase C from WRK1 cell: sensitivity to guanylnucleotides and bacterial toxins. Cell Signal., 1, 541-552.
39. Martin TF. Kowalchyk JA. (1989) Reconstitution of a solubilized membrane but not cytosolic phospholipase C with membrane-associated Gp from GH3 cells. J. Biol. Chem., 264, 20917-20922.
40. Renard D. Poggioli J. Berthon B. Claret M. (1987) How far does phospholipase C activity depend on the cell calcium concentration? Biochem. J. 243, 391-398.
41. Tsien RY. Harootunian AT. (1990) Practical design criteria for a dynamic ratio imaging system. Cell Calcium 11, 93-109.
42. Dudman CC. Reese CB. (1982) Preparation of aryldiazoalkanes from 2,4,6-triisopropylbenzenesulphonyl hydrazones. Synthesis, 1982, 419-421.
43. Nahorski SR. Potter BVL. ( ) Molecular recognition of inositol polyphosphates by intracellular receptors and metabolic enzymes. Trends Pharmacol. Sci., 10, 139-144.
44. Taylor CW. Berridge MJ. Cooke AM. Potter BVL. (1989) Inositol 1,4,5-trisphosphorothioate, a stable analogue of inositol trisphosphate which mobilizes intracellular calcium. Biochem. J., 259, 645-50.
45. DeLisle S. Krause KH. Denning G. Potter BVL. Welsh MJ. (1990) Effect of inositol trisphosphate and calcium on oscillating elevations of intracellular calcium in Xenopus oocytes. J. Biol. Chem., 265, 11726-11730.
46. Berridge MJ. Potter BVL. (1990) Inositol trisphosphate analogues induce different oscillatory patterns in *Xenopus* oocytes. Cell Regul., 1, 675-681.
47. Thastrup O. (1990) Role of Ca^{2+}-ATPases in regulation of cellular Ca^{2+} signalling, as studied with the selective microsomal Ca^{2+}-ATPase inhibitor, thapsigargin. Agents Actions, 29, 8-15.
48. Thastrup O. Cullen PJ. Drobak BK. Hanley MR. Dawson AP. (1990) Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase. Proc. Natl. Acad. Sci. USA, 87, 2466-2470.
49. Law GJ. Pachter JA. Thastrup O. Hanley MR. Dannies PS. (1990) Thapsigargin, but not caffeine, blocks the ability of thyrotropin-releasing hormone to release Ca^{2+} from an intracellular store in GH4C1 pituitary cells. Biochem. J., 267, 359-364.
50. Ganong BR. Loomis CR. Hannun YA. Bell RM. (1986) Specificity and mechanism of protein kinase C activation by *sn*-1,2-diacylglycerols. Proc. Natl. Acad. Sci. USA, 83, 1184-1188.
51. Nerbonne JM. (1986) Design and application of photolabile intracellular probes. In: DeWeer P. Salzberg B.N. eds. Optical Methods in Cell Physiology. New York, Wiley-Interscience, pp 417-445.
52. Miyazaki S. (1988) Inositol 1,4,5-trisphosphate-induced calcium release and guanine nucleotide-binding protein-mediated periodic calcium rises in golden hamster eggs. J. Cell Biol., 106, 345-353.
53. Woods NM. Dixon CJ. Cuthbertson KSR. Cobbold PH. (1990) Fluoroaluminate mimics agonist application in single rat hepatocytes. Biochem. J., 265, 613-615.
54. Rooney TA. Sass EJ. Thomas AP. (1990) Agonist-induced cytosolic calcium oscillations originate from a specific locus in single hepatocytes. J. Biol. Chem., 265, 10792-10796.
55. Chen CF. Corbley MJ. Roberts TM. Hess P. (1988) Voltage-sensitive calcium channels in normal and transformed 3T3 fibroblasts. Science, 239, 1024-1026.
56. Saez JC. Connor JA. Spray DC. Bennett MVL. (1989) Hepatocyte gap junctions are permeable to the second messenger, inositol 1,4,5-trisphosphate, and to calcium ions. Proc. Natl. Acad. Sci. USA, 86, 2708-2712.
57. Sanderson MJ. Charles AC. Dirksen ER. (1990) Mechanical stimulation and intercellular communication increases intracellular Ca^{2+} in epithelial cells. Cell Regul., 1, 585-596.

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