Control of InsP_3-induced Ca^{2+} oscillations in permeabilized blowfly salivary gland cells: contribution of mitochondria

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1. Many agonists linked to the generation of inositol 1,4,5-trisphosphate (InsP_3) and release of Ca^{2+} from intracellular stores induce repetitive transients in cytosolic Ca^{2+} whose frequency increases over a certain range of agonist concentrations.

2. In order to investigate the mechanisms underlying this frequency modulation, the fluorescent Ca^{2+} sensor mag-fura-2 was loaded into intracellular calcium stores and used to monitor InsP_3-induced dynamics of the intraluminal calcium concentration ($[Ca^{2+}]_L$) in secretory cells of permeabilized blowfly *Calliphora vicina* salivary glands.

3. In this preparation, increasing concentrations of InsP_3 induced graded decreases in $[Ca^{2+}]_L$ that were often superimposed with repetitive $[Ca^{2+}]_L$ transients produced by sequential Ca^{2+} release and re-uptake. These $[Ca^{2+}]_L$ oscillations developed at frequencies of 3–11 min^{-1} unrelated to the concentration of InsP_3 present.

4. In contrast, incremental concentrations of InsP_3 applied in the presence of the oxidizable mitochondrial substrates citrate, succinate, or pyruvate–malate induced repetitive $[Ca^{2+}]_L$ transients whose frequency increased with the concentration of InsP_3.

5. This InsP_3 concentration-dependent modulation of oscillation frequency was abolished after dissipating the mitochondrial membrane potential ($\Delta\psi_m$) by combined treatment with carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone + oligomycin or after application of Ruthenium Red, an inhibitor of mitochondrial Ca^{2+} uptake.

6. Taken together, the data indicate that energized mitochondria exert negative control over the frequency of InsP_3-induced Ca^{2+} oscillations. It is concluded that mitochondria play a crucial role in determining the duration of the interspike period and, therefore, for the encoding of amplitude-modulated, InsP_3-liberating stimuli into the frequency of cytosolic Ca^{2+} oscillations.

The activation of receptors coupled to the phosphoinositide pathway induces changes in the free cytosolic calcium concentration ($[Ca^{2+}]_C$) that are generated by release of Ca^{2+} from intracellular stores. These dynamics are often temporally and spatially complex. In many electrically non-excitable cells, inositol 1,4,5-trisphosphate (InsP_3) causes repetitive $[Ca^{2+}]_C$ transients, which are also termed Ca^{2+} oscillations. Locally detected $[Ca^{2+}]_C$ transients often represent Ca^{2+} waves that propagate coordinatedly through individual cells and between clusters of cells coupled by gap junctions. In most of the cells studied so far, the frequency at which consecutive Ca^{2+} spikes are triggered increases with the concentration of the extracellular stimulus or InsP_3 introduced into a cell. This property of the InsP_3/Ca^{2+} second messenger system is assumed to provide the basis for a frequency encoding of external signals (Rapp & Berridge, 1981; Berridge, 1993). Several functional advantages of this signalling mode over amplitude modulated signals have been put forward; among these are an improved signal-to-noise relationship and the avoidance of an adaptation of Ca^{2+}-dependent targets (Meyer & Stryer, 1991; Thomas *et al.* 1996). Furthermore, this property might allow the selective activation of different cellular responses by using Ca^{2+} as a multifunctional messenger. In support of this view, recent experimental evidence suggests that Ca^{2+}-dependent enzymes and transcription factors are differentially tuned to and activated by trains of Ca^{2+} transients of certain frequencies (De Koninck & Schulman, 1998; Dolmetsch *et al.* 1998; Li *et al.* 1998).

Several divergent models have been developed to account for Ca^{2+} oscillations on the basis of extensive experimental and theoretical work (reviewed in Fewtrell, 1993). They have been proposed to be the result of the InsP_3-induced cycling of Ca^{2+} between Ca^{2+}-sensitive and Ca^{2+}-insensitive stores (Goldbeter *et al.* 1990) or, alternatively, to periodic changes in the concentration of InsP_3 caused by feedback of Ca^{2+} on

the activity of phospholipase C (Meyer & Stryer, 1991). Several more recent models have incorporated the modulatory effects of Ca^{2+} on the InsP_3 receptor itself (Bezprozvanny *et al.* 1991; Finch *et al.* 1991). According to these latter models, the rapid upstroke of a Ca^{2+} spike is mediated by feed-forward activation of the InsP_3 receptor by released Ca^{2+}. The termination of Ca^{2+} release and subsequent fall of $[Ca^{2+}]_C$ to basal levels are thought to be attributable to intrinsic or Ca^{2+}-dependent inactivation of the InsP_3 receptor enabling sarco-endoplasmic reticulum-type Ca^{2+}-ATPases to restore basal Ca^{2+} levels by pumping Ca^{2+} back into the InsP_3-sensitive Ca^{2+} stores. Alternatively, the termination of Ca^{2+} spikes is proposed to result from a transient emptying of the Ca^{2+} stores or to be mediated by periodic Ca^{2+}-dependent changes of InsP_3 receptor sensitivity toward InsP_3 (Oancea & Meyer, 1996). In addition to causing the falling phase of individual Ca^{2+} spikes, Ca^{2+}-dependent negative-feedback mechanisms have also been implicated in controlling spike frequency. Thus, the interspike interval has been suggested to reflect the time required by the InsP_3-receptor to recover from inactivation or desensitization (De Young & Keizer, 1992; Atri *et al.* 1993; Oancea & Meyer, 1996).

There is accumulating evidence that mitochondria contribute to the regulation of the dynamics of cytosolic Ca^{2+} signals. In many cells, mitochondria are found in close apposition to the endoplasmic reticulum (ER; Rizzuto *et al.* 1998), and it has been shown that Ca^{2+} released from the ER by InsP_3 can be taken up by mitochondria through a process driven by the mitochondrial-membrane potential and mediated by an electrogenic uniport (Nicholls & Crompton, 1980; Rizzuto *et al.* 1993, 1994, 1999). Based on these morphological and physiological findings, the opening of Ca^{2+} release channels has been suggested to expose mitochondria to microdomains of high $[Ca^{2+}]$, which in turn promotes mitochondrial Ca^{2+} uptake. Thus, mitochondria could act as Ca^{2+} sinks and potentially affect both the amplitude and the time course of InsP_3-induced $[Ca^{2+}]_C$ changes. Indeed, a series of recent studies has demonstrated that experimental activation of mitochondrial metabolism or inhibition of mitochondrial activity causes changes in InsP_3-mediated spatio-temporal $[Ca^{2+}]_C$ dynamics underlining the close functional coupling between InsP_3-dependent Ca^{2+} release and mitochondrial uptake (Jouaville *et al.* 1995; Budd & Nicholls, 1996; Simpson & Russell, 1996; Babcock *et al.* 1997; Boitier *et al.* 1999; Hajnóczky *et al.* 1999).

In previous studies, we have shown that the secretory cells of the intact salivary gland of the blowfly respond to stimulation with the neurohormone 5-hydroxytryptamine (5-HT) or intracellular injections of InsP_3 with repetitive $[Ca^{2+}]_C$ spikes (Zimmermann & Walz, 1997, 1999). Spike frequency has been found to be positively related to the strength of the stimulus. The aim of the present study has been to investigate further the mechanisms controlling the temporal pattern of the agonist-induced $[Ca^{2+}]_C$ dynamics, especially oscillation frequency. To this end, we used a permeabilized preparation in which the $[Ca^{2+}]$ in InsP_3-sensitive Ca^{2+} stores (the endoplasmic reticulum) and the 'cytosol' could be monitored during the direct application of InsP_3.

METHODS

Solutions and media

Ringer solution. *Calliphora* Ringer solution contained (mM): NaCl, 128; KCl, 10; $CaCl_2$, 2; $MgCl_2$, 2; sodium glutamate, 2·7; malic acid, 2·7; Tris, 10; and D-glucose, 10; pH 7·2.

Intracellular-like media (ICM). Calcium sponge S-treated medium was prepared by passing a solution containing (mM): KCl, 125; NaCl, 20; Na_2ATP, 3; Hepes, 10 at pH 7·3 through a column containing ~1 g of calcium sponge S (Molecular Probes, Leiden, Netherlands). $MgCl_2$ was subsequently added to the solution to yield a final concentration of 2 mM. Between passages, the column was regenerated by washing with 100 ml 125 mM KCl, 20 mM NaCl, 10 mM Hepes, pH 4·0 (Tanimura & Turner, 1996). ICM-Chelex was prepared by incubating solutions composed of 125 mM KCl, 20 mM NaCl, 3 mM ATP, 10 mM Hepes at pH 7·3 with 5 g Chelex 100 (Sigma, Deisenhofen, Germany) for ~60 min. After removal of the exchanger resin, $MgCl_2$ was added to give a final concentration of 2 mM. The free Ca^{2+} concentration of media treated with Calcium Sponge S or Chelex was 200–300 nM as determined by Ca^{2+}-sensitive minielectrodes (sensor: ETH129, Fluka, Buchs, Switzerland).

Unless noted otherwise, ICM-EGTA contained (mM): KCl, 125; NaCl, 20; $MgCl_2$, 2; Na_2ATP, 3; EGTA, 0·1; $CaCl_2$, 0·06; Hepes, 10 at pH 7·3. When ICM-EGTA was supplemented with the mitochondrial substrates citric acid (10 mM), succinic acid (10 mM), or pyruvic acid–malic acid (5 mM each), KCl was reduced to 100 mM. The free $[Ca^{2+}]$ of these media was ~250 nM as determined either with Ca^{2+}-selective minielectrodes or fluorometrically with fura-2 (1 μM free acid) by using solutions with defined free-Ca^{2+} concentrations from Molecular Probes (Leiden, Netherlands) as calibration standards.

Blowflies, *Calliphora vicina*, were reared at the Institute. They were kept at 24–26 °C under a dark:light cycle of 12:12 h. Salivary glands were dissected under Ringer solution from the abdomina of adult flies 3–14 days after emergence.

Dye loading and cell permeabilization

Loading of fura-2 into cells of the intact salivary gland. For measurements of $[Ca^{2+}]_C$ in intact secretory cells, the glands were loaded for 20–30 min at room temperature with 5 μM fura-2 acetoxymethyl ester (AM) dissolved in Ringer solution. They were subsequently attached to a coverslip coated with Cell Tak (Collaborative Biomedical Products, Bedford, USA). This coverslip formed the bottom of a perfusion chamber that was mounted to the stage of a Zeiss Axiovert 135TV or Axiovert 100 M inverted epifluorescence microscope. Solution changes were accomplished by means of a peristaltic pump that provided a continuous flux of 0·8 ml min^{-1}.

Mag-fura-2 loading and cell permeabilization. Mag-fura-2 was loaded into cells of intact salivary glands by a 20 min incubation with 5 μM mag-fura-2 AM in Ringer solution. After mounting in the superfusion chamber, glands were briefly washed with ICM and permeabilized for 4–8 min with 50 μg ml^{-1} (w/v) β-escin in ICM, before excessive β-escin was removed with ICM. Permeabilization was monitored by following the washout of cytosolic indicator, i.e. monitoring the fall of mag-fura-2 fluorescence (excited at 340 and

380 nm and detected at 515–565 nm). As a result of the permeabilization, the fluorescence emitted from all the cells within the field of view fell to less than 20 % of its initial intensity.

Loading permeabilized cells with Calcium Green C_{18}. In experiments utilizing Calcium Green C_{18}, cells were first loaded with mag-fura-2 and permeabilized as described. Subsequently, they were incubated for 5 min with 10 μM Calcium Green C_{18}, and washed with ICM until the Calcium Green C_{18} signal (excited at 490 nm) had fallen to a stable level.

TMRE loading. Tetramethylrhodamine ester (TMRE), a lipophilic cationic dye, enters mitochondria in a membrane potential ($\Delta\psi_m$)-dependent manner. Accumulation of the dye causes quenching of fluorescence, whereas mitochondrial depolarization is followed by the release of dye, dequenching and an increase in fluorescence. Changes in TMRE fluorescence can therefore be used to monitor $\Delta\psi_m$. TMRE and mag-fura-2 were coloaded into intact cells by 20 min incubation with 5 μM mag-fura-2 AM and 0·5 μM TMRE in Ringer solution. Subsequently, the cells were permeabilized as described above and further incubated in the presence of 0·1 μM TMRE. Monitoring of the mag-fura-2 fluorescence signals verified successful permeabilization prior to assessing the effects of mitochondrial inhibitors.

Digital fluorescence imaging of Ca^{2+} and $\Delta\psi_m$

Digital Ca^{2+} imaging was essentially carried out as previously described (Zimmermann & Walz, 1997, 1999). Briefly, fura-2 and mag-fura-2 were alternately excited through a × 20 Fluar, 0·75 NA objective at wavelengths 340 and 380 nm provided by a Xenon arc lamp and/or monochromator unit (Till Photonics, Tamm, Germany). Corresponding pairs of fluorescence images taken at 1–2 s intervals were band pass filtered at 515–565 nm, captured by a CCD camera (TE/CCD-512EFT, Princeton Instruments Corp., Trenton, USA) digitized, and transferred to a personal computer for offline processing with the imaging software Metafluor 2.75 (Universal imaging Corp., West Chester, USA). Pairs of fluorescence emission signals (at 515–565 nm) were used to calculate mag-fura-2 ratios. For simultaneous recording of mag-fura-2 and Calcium Green C_{18} fluorescence, the excitation light was sequentially switched between 340, 380 (for mag-fura-2 excitation), and 490 nm (Calcium Green C_{18} excitation). Calcium Creen C_{18} fluorescence was used as a qualitative measure of near-membrane $[Ca^{2+}]$ changes in the permeabilized cytosolic compartment. Fura-2 ratios were calculated after the subtraction of background fluorescence determined at the end of each experiment by quenching fura-2 fluorescence with $MnCl_2$ (Zimermann & Walz, 1997). TMRE fluorescence was excited at 549 nm and detected after passage through a 590 nm long-pass filter.

A single representative region of interest of 19 × 19 μm was selected and used for quantitative evaluations in each preparation. Space–time plots of the $[Ca^{2+}]_L$ dynamics were constructed from series of mag-fura-2 ratio images by sequentially extracting the fluorescence ratio along a line of 1 × 102 pixels (3·8 μm × 385 μm) projected onto the salivary epithelium.

Each trace shown is a representative of at least three independent experiments.

Chemicals

Chemicals were purchased from the following suppliers: fura-2 AM, fura-2 free acid, mag-fura-2 AM, Calcium Green C_{18}, tetramethylrhodamine ester (TMRE) and Calcium Sponge S from Molecular Probes (Leiden, Netherlands); D-myo-inositol 1,4,5-trisphosphate was from Alexis (Grünberg, Germany), ionomycin from Calbiochem (Bad Soden, Germany), and carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone (FCCP), oligomycin, and Ruthenium Red from Sigma (Deisenhofen, Germany). All other chemicals were of analytical grade.

Statistical analysis

Data are expressed as means ± S.D. Statistical comparisons were made by an independent Student's *t* test. *P* values of less than 0·05 were considered as being significant.

RESULTS

Ca^{2+} oscillations in intact cells

Stimulation of the intact blowfly salivary gland with subsaturating concentrations of 5-HT causes $[Ca^{2+}]_C$ oscillations that are generated by periodic Ca^{2+} fluxes out of and into intracellular Ca^{2+} stores. Oscillation frequency rises from ~1 min^{-1} at threshold [5-HT] to ~7 min^{-1} as the strength of the stimulus is increased (Fig. 1; see also Zimmermann & Walz, 1997).

Ca^{2+} oscillations in β-escin-permeabilized cells

In order to gain experimental access to the intracellular Ca^{2+} stores and to directly control the cytosolic factors that contribute to the spatio-temporal characteristics of Ca^{2+} oscillations we used β-escin-permeabilized secretory cells and the fluorescent low-affinity Ca^{2+}-indicator mag-fura-2, which has been reported to accumulate in $InsP_3$-sensitive Ca^{2+} stores (Hofer & Machen, 1993; Chatton *et al.* 1995). As shown in Fig. 2*A*, the addition of $InsP_3$ to permeabilized, mag-fura-2-loaded secretory cells bathed in artificial intracellular medium (ICM, supplemented with 1 mM EGTA) caused concentration-dependent sustained decreases of the fluorescence ratio. These declines followed simple kinetics and were completely reversed by the addition of heparin, a competitive inhibitor of $InsP_3$-mediated Ca^{2+} release, demonstrating that the indicator indeed monitored $[Ca^{2+}]$ changes in $InsP_3$-sensitive Ca^{2+} stores (Fig. 2*B*). In contrast to $InsP_3$, application of caffeine (10 or 25 mM), a substance

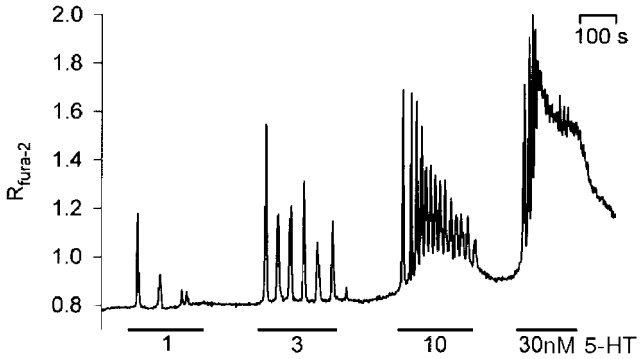

Figure 1. Concentration–response relationship of 5-hydroxytryptamine (5-HT)-induced $[Ca^{2+}]_C$ responses in the intact salivary gland

Stimulation of glands loaded with fura-2 elicits repetitive $[Ca^{2+}]_C$ spikes whose frequency increases with the concentration of 5-HT applied. Duration of individual stimulations and concentrations of the agonist are indicated by horizontal bars and numbers, respectively.

that induces Ca^{2+} release through ryanodine receptors did not cause decreases in the mag-fura-2 signal. This result is in line with the findings of an earlier study in intact glands (Zimmermann & Walz, 1997) which also failed to produce evidence for ryanodine receptors in this type of cells. Likewise, addition of the mitochondrial uncoupler FCCP (1 μM) and oligomycin (2·5 μg ml^{-1}) to permeabilized gland cells did not cause any detectable change in the mag-fura-2 ratio ($n = 3$), indicating that mag-fura-2, under these conditions, did not detect $[Ca^{2+}]$ changes within mitochondria (*compare* Hofer & Machen, 1994).

When added to ICM pretreated with Chelex 100 or Calcium Sponge S, or to ICM supplemented with only 100 μM EGTA, InsP_3 induced tonic declines of $[Ca^{2+}]_L$ that were often superimposed with repetitive Ca^{2+} transients consisting of a rapid initial decrease (time to reach the inverted peak < 4 s) that was followed by a slower recovery (Fig. 3*A*). Thus, when applied in ICM in which Ca^{2+} was only weakly buffered, InsP_3 evoked $[Ca^{2+}]_L$ oscillations that were inverted relative to the $[Ca^{2+}]_C$ oscillations elicited by 5-HT or InsP_3 in intact cells. This suggested that $[Ca^{2+}]_C$ changes were necessary for the $[Ca^{2+}]_L$ oscillations to occur.

To record $[Ca^{2+}]_L$ oscillations and $[Ca^{2+}]$ simultaneously in the cytosolic compartment, mag-fura-2-loaded permeabilized cells were briefly incubated with Calcium Green C_{18}, a Ca^{2+} indicator whose lipophilic alkyl side chain intercalates into lipid membranes. Calcium Green C_{18} can therefore be used to monitor $[Ca^{2+}]$ in the vicinity of membranes (Lloyd *et al.*

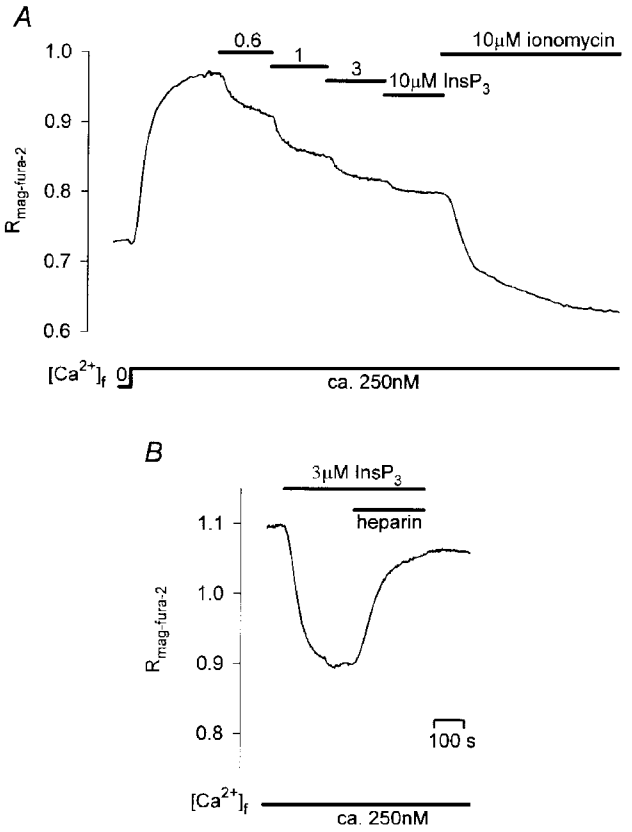

Figure 2. Effects of Ca^{2+}, InsP_3 and heparin on $[Ca^{2+}]_L$
A, following permeabilization in Ca^{2+}-free ICM, addition of Ca^{2+} to ICM-1 mM EGTA (1 mM EGTA, 600 μM $CaCl_2$, $[Ca^{2+}]_{free}$, 250 nM) caused rises in the mag-fura-2 ratio indicative of Ca^{2+} uptake into Ca^{2+}-storing organelles. The subsequent incremental addition of InsP_3 resulted in graded declines of $[Ca^{2+}]_L$. *B*, inhibition of InsP_3-induced Ca^{2+} release by heparin (1 mg ml^{-1}). Additions were made as indicated. The traces shown in *A* and *B* are representative of 6 and 3 independent experiments, respectively.

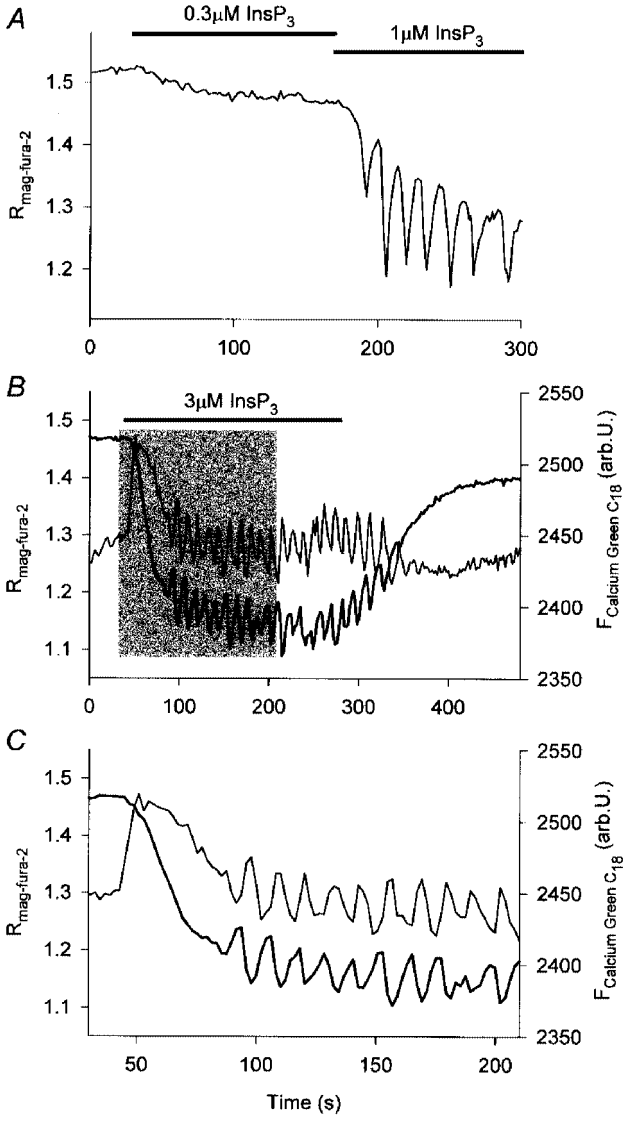

Figure 3. Effects of InsP_3 on $[Ca^{2+}]_L$ and near-membrane $[Ca^{2+}]_C$ of permeabilized salivary gland cells bathed in ICM-Chelex
A, changes in $[Ca^{2+}]_L$ induced by InsP_3. Under these conditions, the addition of InsP_3 resulted in graded declines in $[Ca^{2+}]_L$ often accompanied by repetitive $[Ca^{2+}]_L$ transients. *B* and *C*, $[Ca^{2+}]_L$ (thick line) and near-membrane $[Ca^{2+}]_C$ (thin line) monitored simultaneously with mag-fura-2 and Calcium Green C_{18}, respectively. *C* shows part of the traces marked by the shaded area in *B* on an extended time scale. InsP_3 (3 μM) was added as indicated.

Table 1. Apparent EC_{50} of $InsP_3$ on sustained declines in $[Ca^{2+}]_L$ under various experimental conditions

	EGTA 1 mM	Chelex	EGTA 100 μM	EGTA-citrate	EGTA-succinate	EGTA-pyruvate/malate
Control (μM)	2·5	1·3	1·7	1·0	1·3	2·6
FCCP–oligomycin (μM)	n.d.	n.d.	n.d.	1·3	n.d.	2·1

EC_{50} values were determined by a least squares fit of data pooled from 6 to 16 independent experiments to the Hill equation (as shown in Figs 5*A* and 8*A*).

1995). In these experiments, mag-fura-2 and Calcium Green C_{18} reported temporally tightly coupled complementary $[Ca^{2+}]$ changes in response to the addition of $InsP_3$ (Fig. 3*B* and *C*) consistent with an $InsP_3$-induced periodic Ca^{2+} exchange between Ca^{2+} stores and cytosol. Most notably, the temporal evolution of $[Ca^{2+}]_C$ monitored by Calcium Green C_{18} was remarkably similar to $[Ca^{2+}]_C$ changes elicited by 5-HT or $InsP_3$ in intact cells (cf. Fig. 2*B* and *C* in Zimmermann & Walz, 1999). Taken together, these findings demonstrated that the permeabilized system reproduced key parameters of $InsP_3$-dependent Ca^{2+} signalling previously described in intact cells (see also Tanimura & Turner, 1996; Hajnóczky & Thomas, 1997).

In order to establish the relationship between $InsP_3$ concentration and the threshold, the amplitude and the frequency of these $[Ca^{2+}]_L$ oscillations, $InsP_3$ was next incrementally varied from 0 to 10 μM. Such stepwise elevations of $[InsP_3]$ in either ICM-Chelex or ICM-100 μM EGTA caused gradual tonic decreases in $[Ca^{2+}]_L$ whose amplitudes were similar to the respective decreases elicited in the presence of 1 mM EGTA (Figs 4 and 5*A*; Table 1). As shown in Fig. 4, sustained decreases in $[Ca^{2+}]_L$ were frequently accompanied by rapid $[Ca^{2+}]_L$ oscillations, with an increasing fraction of preparations displaying oscillations when $[InsP_3]$ was raised from 0·3 to 3 μM (Fig. 5*B*). Surprisingly, $[Ca^{2+}]_L$ oscillation frequencies appeared to be little affected by the concentration of $InsP_3$ present. Statistical comparison of the experimental data confirmed that there was no significant dependence of absolute or relative oscillation frequencies on $[InsP_3]$ (Fig. 5*C*, Table 2). Like oscillation frequency, $[Ca^{2+}]_L$-spike amplitudes showed little change with changes in $[InsP_3]$ in most of the preparations studied. Only in a few experiments did spike amplitudes increase when $InsP_3$ was varied between 0·3 and 1 μM. Most notably, changes in amplitude were not accompanied by concomitant changes in oscillation frequency (Fig. 4*B*). In summary, application of different concentrations of $InsP_3$ to permeabilized cells failed to duplicate the prominent concentration-dependent frequency modulation of $InsP_3$-mediated $[Ca^{2+}]_C$ oscillations in intact cells (cf. Fig. 1). In particular, low frequency oscillations ($f < 3\ min^{-1}$) generated by low agonist concentrations in intact cells were not observed.

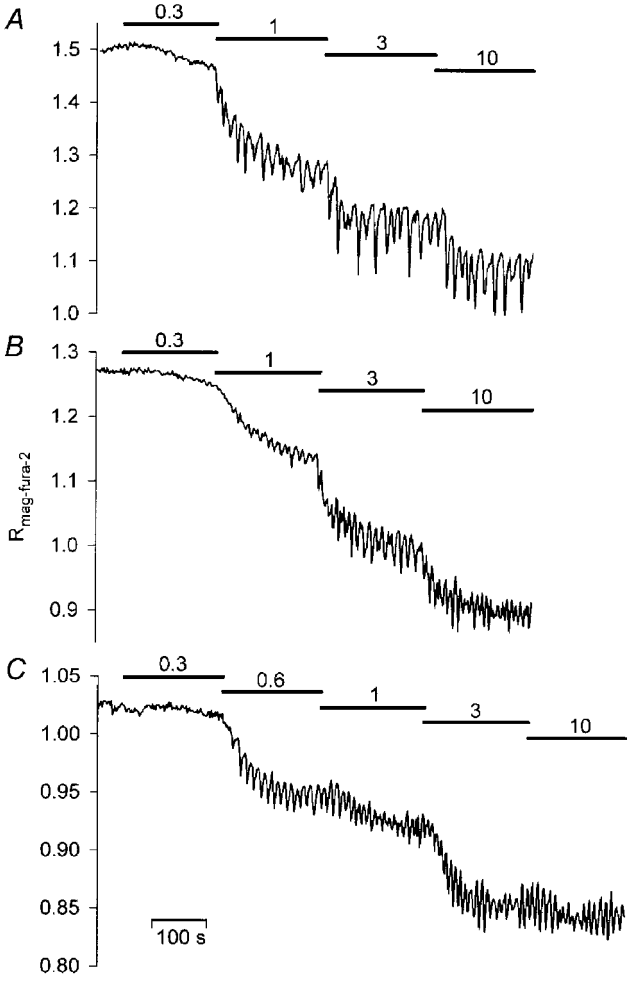

Figure 4. Dependence of changes in $[Ca^{2+}]_L$ on the concentration of $InsP_3$ added to weakly Ca^{2+}-buffered media

$InsP_3$ was applied to ICM pretreated with Chelex (*A*) or supplemented with 100 μM EGTA (*B* and *C*). Stepwise increases in $InsP_3$ elicited sustained decreases in $[Ca^{2+}]_L$ and $[Ca^{2+}]_L$ oscillations, whose frequencies are apparently independent of the $[InsP_3]$ present. $InsP_3$ was added as indicated by the horizontal bars, numbers indicate the respective $InsP_3$ concentration in μM.

Contribution of mitochondrial activity to InsP_3-evoked Ca^{2+} oscillations in permeabilized cells

Earlier studies have demonstrated that the frequency of agonist- and InsP_3-evoked Ca^{2+} oscillations is sensitive to changes in the mitochondrial membrane potential ($\Delta\psi_m$) (Jouaville *et al.* 1995). Thus, we hypothesized that the apparent loss of frequency modulation after cell permeabilization might be the result of impaired mitochondrial function and therefore, attempted to test whether increasing $\Delta\psi_m$ by the addition of oxidizable mitochondrial substrates affected the InsP_3-evoked $[Ca^{2+}]_L$ dynamics. Effects of tricarboxylic acid cycle substrates on $\Delta\psi_m$ were first directly examined by monitoring the fluorescence of TMRE, a cationic fluorescent indicator that exhibits a Nernstian distribution across the inner mitochondrial membrane and is subject to self-quenching at high concentrations. To this end, glands were loaded with TMRE, permeabilized and first incubated in ICM supplemented with the mitochondrial substrate citrate (10 mM). Subsequently citrate was removed from the bathing medium. This resulted in a large increase in TMRE fluorescence which could be fully reversed by re-administrating citrate (Fig. 6*A*; $n = 6$). These changes in the fluorescence of TMRE are indicative of the release and re-uptake of dye from and into mitochondria, i.e. a

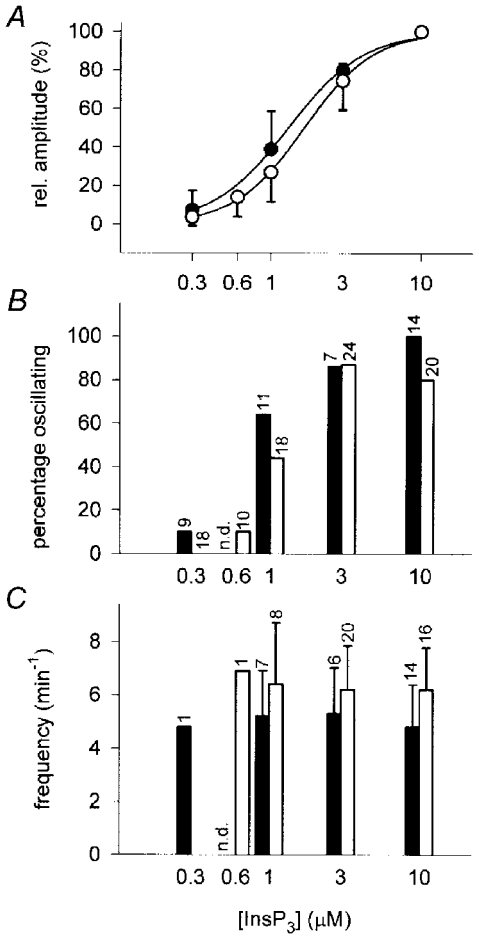

Figure 5. Concentration dependence of amplitude, threshold and frequency of InsP_3-induced changes in $[Ca^{2+}]_L$

A, relative amplitudes of InsP_3-induced persistent falls of the mag-fura-2 ratio in response to the application of InsP_3 in ICM-Chelex (●) and ICM-100 μM EGTA (○). Amplitudes were determined from traces as shown in Fig. 4, 3 min after the addition of the respective concentration of InsP_3. Amplitudes of declines at 10 μM InsP_3 were set to 100 %. Lines indicate the best least squares fits of the data to the Hill function. *B*, fraction of preparations exhibiting $[Ca^{2+}]_L$ oscillations at a given InsP_3 in ICM-Chelex (filled bars) or ICM-100 μM EGTA (open bars). Numbers on top of the bars indicate the total number of preparations studied. Preparations that did not show oscillations at any of the concentrations tested were excluded. *C*, frequencies of Ca^{2+} oscillations at various [InsP_3] in ICM-Chelex (filled bars) and ICM-100 μM EGTA (open bars). Numbers on top indicate the number of preparations exhibiting oscillations (preparations that did not show $[Ca^{2+}]_L$ oscillations at the respective [InsP_3] were not included). Data were extracted from experiments representatively depicted in Fig. 4.

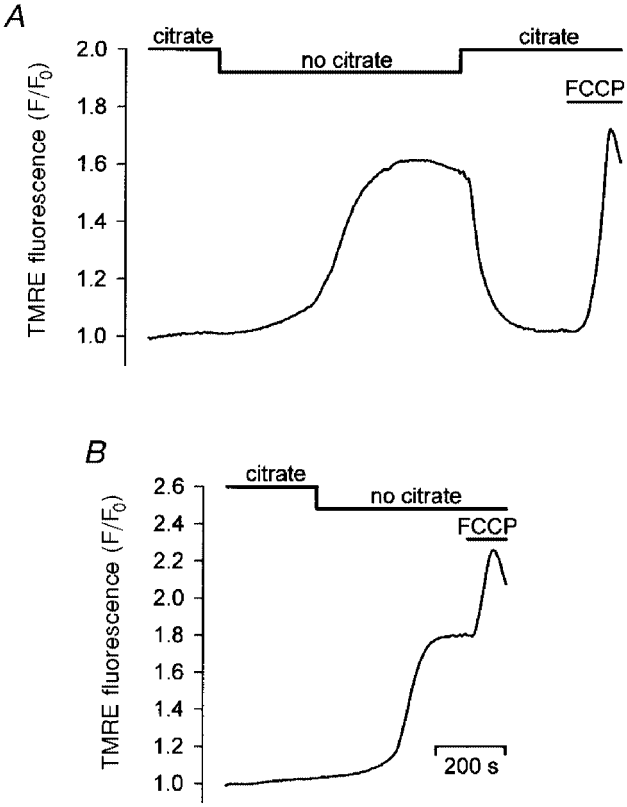

Figure 6. Effect of oxidizable mitochondrial substrates on the mitochondrial membrane potential

A, glands were permeabilized in ICM-citrate. Removal of citrate from the incubation medium was followed by an increase in TMRE fluorescence, indicative of mitochondrial depolarization. TMRE fluorescence decreased to its initial level after readdition of citrate. Addition of FCCP (1 μM) to dissipate $\Delta\psi_m$ at the end of the experiment caused a rapid rise of the signal. *B*, withdrawal of citrate from the intracellular-like medium resulted in a persistent rise in TMRE fluorescence. Subsequent dissipation of $\Delta\psi_m$ with FCCP (1 μM) further increased the TMRE signal.

reversible decrease of the mitochondrial membrane potential. Addition of the mitochondrial uncoupler FCCP (1 μM) in citrate-deficient medium was followed by a further increase in TMRE fluorescence showing that removing the mitochondrial substrate from the intracellular-like medium had diminished but not completely dissipated $\Delta\psi_m$ (Fig. 6*B*).

Increasing concentrations of $InsP_3$ applied in the presence of the mitochondrial substrates citrate (10 mM), succinate (10 mM), or pyruvate/malate (5 mM/5 mM) caused changes in $[Ca^{2+}]_L$ that consisted of graded declines and concomitant repetitive transient falls (Fig. 7). These responses were qualitatively similar to the responses observed in the absence of mitochondrial substrates. Plotting of the tonic changes in $[Ca^{2+}]_L$ as a function of $[InsP_3]$ revealed that the decline of $[Ca^{2+}]_L$ was little affected by the addition of substrates (compare Figs 5*A* and 8*A*; Table 1). In contrast to the previous experiments, however: (1) a greater fraction of the preparations showed $[Ca^{2+}]_L$ oscillations at low and

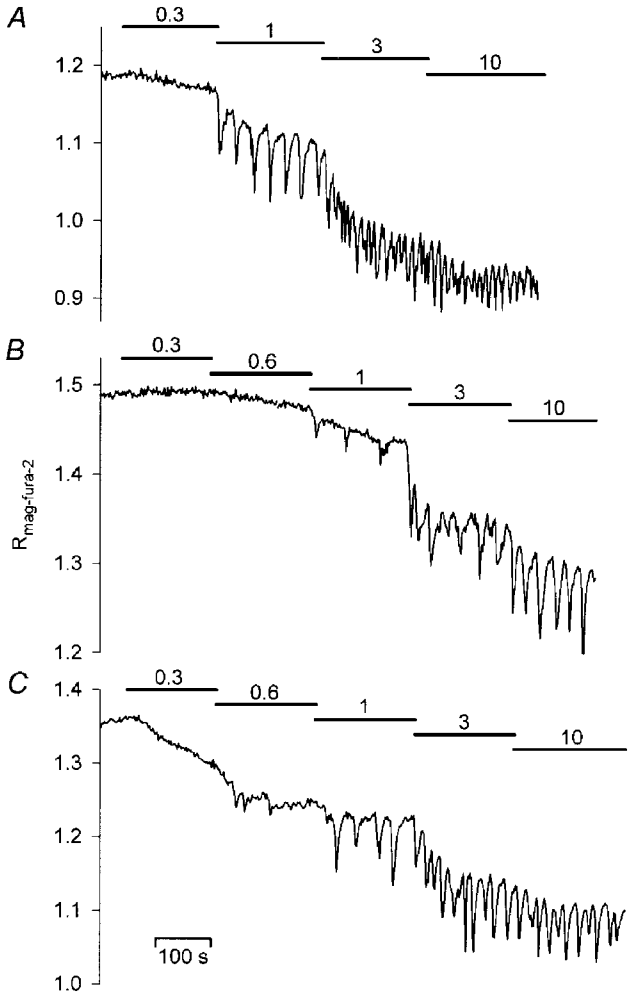

Figure 7. $InsP_3$-induced changes in $[Ca^{2+}]_L$ determined in the presence of oxidizable mitochondrial substrates

Preparations were challenged by step-wise increases in $[InsP_3]$ applied in ICM-citrate (*A*), ICM-succinate (*B*), or ICM-pyruvate/malate (*C*). Under all these conditions, increasing $[InsP_3]$ caused graded declines in $[Ca^{2+}]_L$ accompanied by an increase in the frequency of $[Ca^{2+}]_L$ oscillations. To avoid changes in the free Ca^{2+} concentration of the intracellular-like media resulting potentially from the addition of mitochondrial substrates, these experiments were performed in ICM supplemented with 100 μM EGTA.

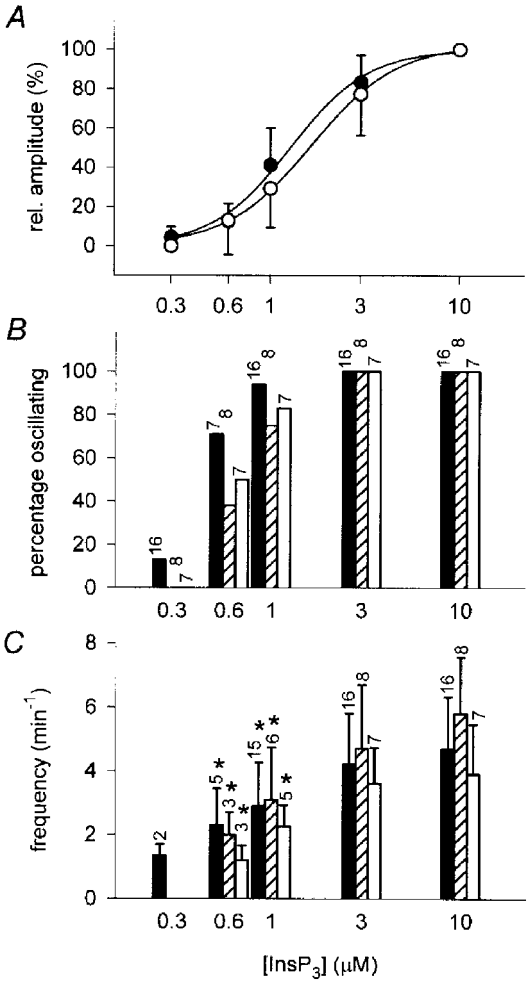

Figure 8. Concentration dependence of amplitude, threshold and frequency of $InsP_3$-induced changes in $[Ca^{2+}]_L$ in the presence of oxidizable mitochondrial substrates

A, relative amplitudes of $InsP_3$-evoked tonic falls in the mag-fura-2 ratio determined in ICM-citrate (●) or ICM-puruvate/malate (○). Amplitudes of declines at 10 μM $InsP_3$ were set to 100 %. Data from experiments performed in ICM-succinate are similar and are not shown for reasons of clarity. *B*, fraction of preparations responding with $[Ca^{2+}]_L$ oscillations to a given $[InsP_3]$ in ICM-citrate (filled bars), ICM-succinate (hatched bars), or ICM-pyruvate/malate (open bars). Numbers above bars indicate the number of experiments included in each data set. *C*, oscillation frequencies at different $[InsP_3]$ in the presence of mitochondrial substrates: citrate (10 mM, filled bars), succinate (10 mM, hatched bars), or pyruvate/malate (5 mM each, open bars). Numbers, as *B*. * Oscillation frequencies that are significantly ($P < 0{\cdot}05$) different from the frequencies at 10 μM $InsP_3$ (see also Table 2). Amplitudes of tonic declines, fractions of responsive preparations, and oscillation frequencies were determined as described in Fig. 5.

Table 2. $[Ca^{2+}]_L$ oscillation frequencies determined at 1 or 10 μM $InsP_3$ in various intracellular-like media and in the presence of mitochondrial inhibitors

Intracellular medium	$InsP_3$ alone			+ FCCP–oligomycin			+ Ruthenium Red		
	1 μM $InsP_3$	10 μM $InsP_3$	P	1 μM $InsP_3$	10 μM $InsP_3$	P	1 μM $InsP_3$	10 μM $InsP_3$	P
Chelex	5·2 ± 1·7 (7)	4·8 ± 1·6 (14)	> 0·5	—	—	—	—	—	—
100 μM EGTA	6·4 ± 2·3 (8)	6·2 ± 1·6 (15)	> 0·7	—	—	—	—	—	—
EGTA-citrate	2·9 ± 1·4 (15)	4·7 ± 1·6 (16)	**< 0·002**	4·7 ± 1·3 (5)	4·4 ± 1·0 (7)	> 0·6	4·5 ± 1·1 (4)	3·7 ± 1·0 (5)	> 0·3
EGTA-succinate	3·1 ± 1·6 (6)	5·8 ± 1·8 (8)	**< 0·01**	—	—	—	—	—	—
EGTA-pyruvate/ malate	2·3 ± 0·7 (5)	3·9 ± 1·6 (7)	**< 0·05**	—	—	—	—	—	—

Oscillation frequencies are given in spikes min^{-1}. Numbers in parentheses indicate the number of experiments performed under each condition. P values indicate the levels of significance with respect to differences in oscillation frequency at 1 and 10 μM $InsP_3$ in a given medium using Student's non-paired t test. Bold type highlights values considered to be significantly different.

intermediate $InsP_3$ concentrations (71 *vs.* 10% at 0·6 μM $InsP_3$ in ICM-citrate and ICM-EGTA, respectively; Fig. 8*B*); (2) transients reached larger amplitudes than in the absence of substrates (Fig. 7) and (3) oscillation frequencies clearly increased as the concentration of $InsP_3$ was raised (Fig. 8*C*, Table 2). Some preparations responded to the addition of 0·3 or 0·6 μM $InsP_3$ with only a single transient during a 3 min stimulation period. Therefore, the lowest frequencies measured in these experiments are likely to overestimate the lower limit of oscillation frequencies. Despite the greater interspike intervals at low and intermediate levels of stimulation, $[Ca^{2+}]_L$ did not

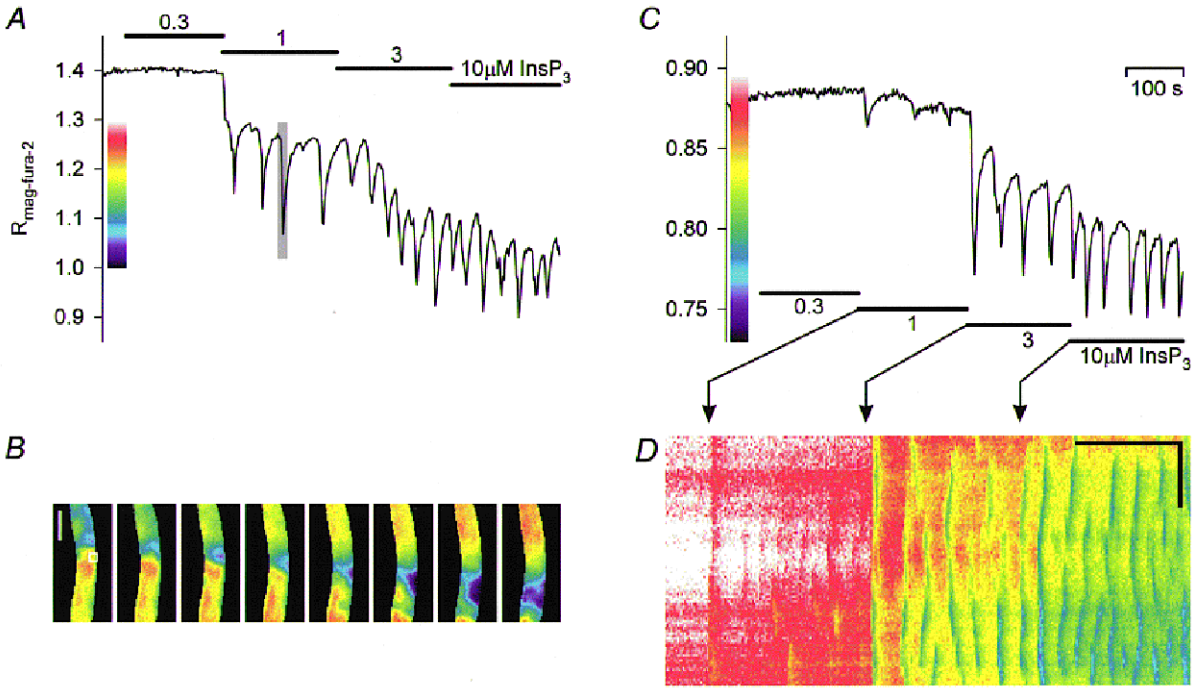

Figure 9. Spatio-temporal pattern of $InsP_3$-mediated $[Ca^{2+}]_L$ oscillations

$InsP_3$ delivered at increasing concentrations in ICM-citrate (*A*) or ICM-pyruvate/malate (*C*). *B*, series of pseudocolour images of the mag-fura-2 fluorescence ratio corresponding to the time segment indicated by the shaded area in *A*. Image intervals 2 s. The lateral diameter of the secretory cells that form the tube-like single-layered epithelium is ~15–20 μm. The white box (19 μm × 19 μm) indicates the region selected to construct the graph in *A*; vertical scale bar 100 μm. *D*, space–time plot of *B*. A line (1 pixel × 102 pixels, 3·8 μm × 385 μm) projected onto the permeabilized gland was sequentially extracted from a series of pseudocolour images of the mag-fura-2 ratio and stacked according to the time of acquisition. Thus, the ordinate of the plot represents the location, whereas the abscissa represents time. Bands of altered pseudocolours in the plot reflect coordinate transient falls in $[Ca^{2+}]_L$. Horizontal scale bar 100 s, vertical scale bar 100 μm.

completely recover to pre-stimulation levels between individual transients (Figs 7, and 9*A* and *C*) indicating that repetitive transient release and continuous Ca^{2+} efflux from the Ca^{2+} stores between spikes contributed to the overall decrease in store Ca^{2+} content.

The low oscillation frequencies observed at low [$InsP_3$] in the presence of mitochondrial substrates allowed the spatio-temporal organization of the intraluminal [Ca^{2+}] changes to be resolved. This analysis revealed coordinated waves of Ca^{2+} release over distances corresponding to the lateral diameters of several cells (Fig. 9*B* and *D*).

The substances used to activate mitochondria are also known to bind Ca^{2+}, though with low affinity. To test whether the effects of the substrates were due to increasing the Ca^{2+} buffer capacity of the media or mitochondrial activation, we investigated the effects of blocking mitochondrial activity while mitochondrial substrates were present. The rationale behind this experiment was that blocking mitochondrial activity should reverse the effects of the mitochondrial substrates while the Ca^{2+}-buffering capacity of the intracellular-like medium should not be changed. To this end, we used FCCP (1 μM) in combination with oligomycin (2·5 μg ml^{-1}), to prevent activation of the mitochondrial ATP synthase in the reverse mode. Pre-incubation with FCCP–oligomycin in ICM-citrate did not measurably affect the amplitudes of $InsP_3$-induced persistent declines of $[Ca^{2+}]_L$ (Table 2), but, as shown in Fig. 10*A* and *D*, the presence of FCCP–oligomycin resulted in the complete loss of the [$InsP_3$]-dependent modulation of Ca^{2+} oscillation frequency and produced oscillations of high frequencies at any concentration of $InsP_3$ tested. Thus, the preparations treated according to this protocol behaved like the preparations stimulated in the absence of mitochondrial substrates.

FCCP and oligomycin can be expected to dissipate mitochondrial proton motive force, $\Delta\psi_m$ and inhibit mitochondrial ATP production. Therefore, the effects of these substances on $InsP_3$-evoked Ca^{2+} oscillations in the salivary gland may have resulted from the inhibition of $\Delta\psi_m$-dependent mitochondrial Ca^{2+} uptake or from local

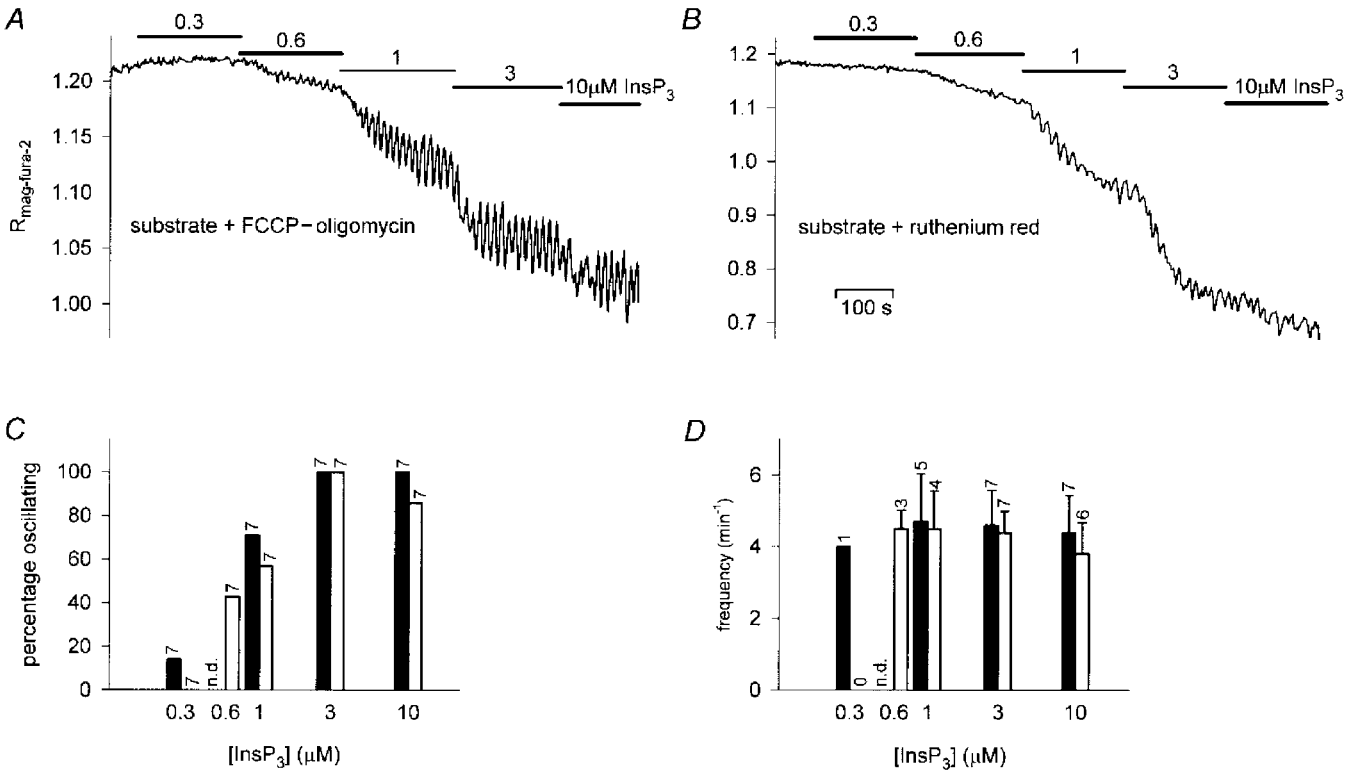

Figure 10. Effects of mitochondrial inhibitors on $InsP_3$-induced dynamics of $[Ca^{2+}]_L$

A, time course of changes of the mag-fura-2 ratio induced by graded increases of [$InsP_3$] in the presence of the mitochdrial uncoupler FCCP (1 μM) and the mitochondrial F_0F_1-ATPase inhibitor oligomycin (2·5 μg ml^{-1}). Both substances were added approximately 5 min before stimulation. The experiment was performed in ICM-citrate. *B*, time course of changes of the mag-fura-2 ratio induced by graded increases of [$InsP_3$] in the presence of Ruthenium Red (10 μM). *C*, fraction of preparations exhibiting $[Ca^{2+}]_L$ oscillations at given $InsP_3$ and FCCP–oligomycin (filled bars, see also *A*) or Ruthenium Red (open bars, see also *B*). Numbers above the bars indicate the total number of preparations studied. Preparations that did not show oscillations at any of the concentrations tested were not included. *D*, frequencies of Ca^{2+} oscillations at different [$InsP_3$] in the presence of FCCP–oligomycin (filled bars) or Ruthenium Red (open bars). Numbers above the bars indicate the number of preparations exhibiting oscillations.

ATP depletion in the vicinity of InsP_3 receptors whose gating has been shown to be sensitive to [ATP] (Smith *et al.* 1985; Ferris *et al.* 1990; Landolfi *et al.* 1998). To discriminate between these potential effects, glands were stimulated with InsP_3 in the presence of citrate (10 mM) and Ruthenium Red, an inhibitor of the mitochondrial Ca^{2+} uniporter. Upon treatment with Ruthenium Red (10 μM) the frequency of the Ca^{2+} oscillations again became independent of the InsP_3 concentration (Fig. 10*B* and *D*; Table 2), suggesting that functional mitochondrial Ca^{2+} uptake was required for the [InsP_3]-dependent modulation of Ca^{2+} oscillation frequency.

To follow the effects of the dissipation of $\Delta\psi_m$ directly in the presence of InsP_3, FCCP and oligomycin were next added after low-frequency oscillations were elicited at a constant intermediate concentration of InsP_3 in ICM-citrate. Following the addition of FCCP–oligomycin, $[Ca^{2+}]_L$ oscillations ceased in three out of six preparations treated according to this protocol (Fig. 11*A*), whereas the frequency in the remaining three experiments increased from $2{\cdot}2 \pm 1{\cdot}30$ to $4{\cdot}7 \pm 0{\cdot}82$ min^{-1} (cf. Fig. 11*B*). This finding shows directly that the dissipation of $\Delta\psi_m$ relieves the negative control of mitochondria over the frequency of Ca^{2+} oscillations and shifts the threshold for the establishment of Ca^{2+} oscillations to lower [InsP_3] as suggested by the previous experiments (compare Figs 5*B* and *C* and 8*B* and *C*). An additional immediate effect of the application of inhibitors in these experiments was, as shown in Fig. 11*A* and *B*, a slow fall of $[Ca^{2+}]_L$ and subsequent recovery. We cannot rule out that this is the result of a direct effect of the inhibitors on InsP_3-sensitive stores rather than on mitochondria. However, such transients were not observed when FCCP–oligomycin were applied in the absence of InsP_3 (see also above), and the dissipation of $\Delta\psi_m$ is known to reverse the activity of the mitochondrial Ca^{2+} uniporter, which then becomes an efflux pathway. Thus, the FCCP–oligomycin-induced transient decreases in $[Ca^{2+}]_L$ may be a consequence of the activation of Ca^{2+} release via InsP_3 receptor channels triggered by Ca^{2+} leaking from depolarized mitochondria.

DISCUSSION

The results of the present study demonstrate that the application of InsP_3 to permeabilized salivary gland cells of the blowfly causes periodic Ca^{2+} release from intracellular Ca^{2+} stores into the cytosol and thereby reproduces hormone-evoked $[Ca^{2+}]_C$ oscillations in the intact gland. Most remarkably, InsP_3-induced oscillatory Ca^{2+} release episodes spread over several neighbouring cells as a coordinated wave, as do cytoplasmic Ca^{2+} oscillations in intact cells.

Mechanism underlying Ca^{2+} oscillations in the permeabilized cells

Ca^{2+} oscillations in the permeabilized preparation did not occur under conditions of strong Ca^{2+} buffering suggesting that changes in $[Ca^{2+}]_C$ are crucial for this process. When buffering of $[Ca^{2+}]_C$ with high concentrations of EGTA prevented Ca^{2+} oscillations, InsP_3 caused declines of $[Ca^{2+}]_L$ whose amplitude increased with the concentration of InsP_3. Under the conditions of these experiments, constant $[Ca^{2+}]_L$ reflects a steady state produced by opposing fluxes of Ca^{2+} out of and into InsP_3-sensitive Ca^{2+} stores. The observed monotonous declines in $[Ca^{2+}]_L$ are, therefore, readily explained by stably increased rates of InsP_3-induced Ca^{2+} release at a constant rate of Ca^{2+} uptake, most likely via SERCA-type Ca^{2+} pumps. Thus, the present findings suggest that increasing concentrations of InsP_3 at clamped $[Ca^{2+}]_C$ recruited an increasing population of InsP_3 receptors, which then provided a steady concentration-dependent flux of Ca^{2+} from the Ca^{2+} stores. A similar pattern of Ca^{2+} release occurred when low Ca^{2+} buffering of the artificial intracellular medium permitted Ca^{2+} oscillations. The tonic falls of $[Ca^{2+}]_L$ observed under these conditions clearly do not result from an incomplete recovery between spikes, because they were also seen under conditions that produced interspike intervals long enough for $[Ca^{2+}]_L$ to recover completely from the phases of enhanced release (see Figs 7 and 9). This implies that Ca^{2+} release induced by InsP_3 in permeabilized salivary gland cells bathed in weakly Ca^{2+}-buffered media consists of two components: (1) periodic

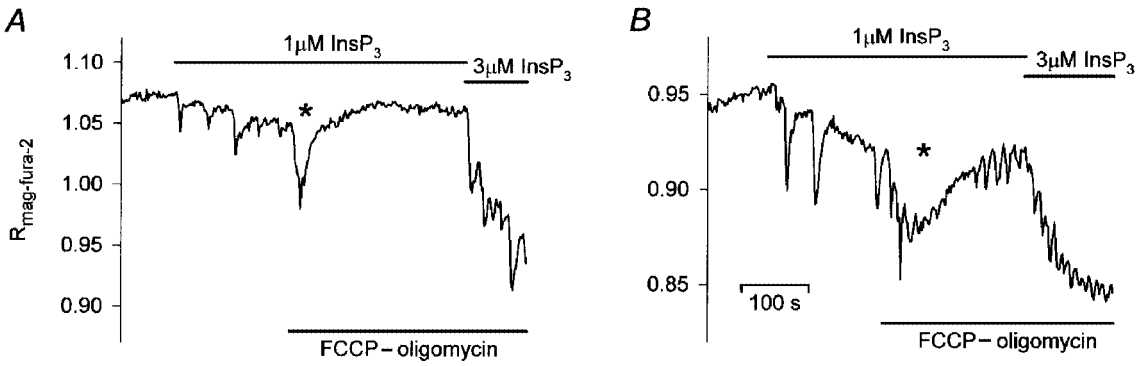

Figure 11. Effects of mitochondrial inhibitors on $[Ca^{2+}]_L$ at constant [InsP_3]

Experiments were carried out in ICM-citrate. Addition of FCCP (1 μM) and oligomycin ($2{\cdot}5$ μg ml^{-1}) was followed by a transient fall in $[Ca^{2+}]_L$ (*) and either stopped the $[Ca^{2+}]_L$ oscillations induced by InsP_3 (1 μM) (*A*) or caused the oscillation frequency to increase (*B*). InsP_3 (3 μM) was added at the end of the experiments to verify the responsiveness of the preparations.

transient Ca^{2+} release events dependent on fluctuations in $[Ca^{2+}]_C$ and (2) an apparently steady Ca^{2+} efflux between Ca^{2+} spikes whose magnitude varies with the concentration of $InsP_3$.

Our data are therefore consistent with a subsaturating concentration of $InsP_3$ causing the opening of a fraction of $InsP_3$ receptor Ca^{2+} channels located in the membranes of intracellular Ca^{2+} stores, thereby creating increased concentration-dependent fluxes of Ca^{2+} into the cytosol. The resultant local elevations in $[Ca^{2+}]_C$ then excite neighbouring $InsP_3$ receptors and induce the regenerative release of additional Ca^{2+}, which underlies the rapid fall in $[Ca^{2+}]_L$ and the upstroke of a $[Ca^{2+}]_C$ spike. This phase of rapid release is probably the result of the well-documented Ca^{2+}-mediated shift in $InsP_3$ receptor sensitivity toward $InsP_3$ (Baumann & Walz, 1989; Parker & Ivorra, 1990; Bezprozvanny *et al.* 1991; Finch *et al.* 1991). Subsequent closure of the channels may be induced by Ca^{2+} feedback inhibition (Iino, 1990; Meyer & Stryer, 1991; Bezprozvanny *et al.* 1991; Finch *et al.* 1991) or by intrinsic inactivation of Ca^{2+}-bound receptors (Hajnóczky & Thomas, 1997). This inactivation, in concert with the activity of SERCA-type Ca^{2+} pumps, is likely to terminate the spike and to lead to a fall of $[Ca^{2+}]_C$ back to baseline. Finally, recovery from inactivation reestablishes the initial sustained $InsP_3$-induced Ca^{2+} fluxes, thereby completing the cycle and resetting the system. According to this sequence of events, Ca^{2+} oscillations are essentially driven by $InsP_3$-evoked Ca^{2+} efflux periodically triggering Ca^{2+}-induced Ca^{2+} release from a single internal Ca^{2+} pool. This model does not invoke periodic changes in concentration of $InsP_3$ and directly explains the spatial spread of Ca^{2+} waves from sites of Ca^{2+} spike initiation (Fig. 9*B* and *D*; De Young & Keizer, 1992; Atri *et al.* 1993).

Mechanisms controlling Ca^{2+} oscillation frequency in the permeabilized cells

A central observation of the present study is that the frequency modulation of Ca^{2+} oscillations observed in intact cells was, in our hands, completely lost after permeabilization of the plasma membranes. This means that whatever the specific mechanism underlying periodic $InsP_3$ receptor Ca^{2+}-release channel closure, it is unlikely to account for the moduation of oscillation frequency. Consistent with this notion, others have found that the recovery of the $InsP_3$ receptor from $InsP_3$- or Ca^{2+}-dependent inactivation proceeds with half-times ranging from one to a few seconds (Ogden *et al.* 1990; Finch *et al.* 1991; Parker *et al.* 1996; Hajnóczky & Thomas, 1997). Thus, recovery from inactivation is too rapid to limit oscillation frequency at low stimulus strengths, when interspike intervals reach 1–2 min in the salivary gland (Zimmermann & Walz, 1997) and up to several minutes in other cells. The present data show that, in permeabilized salivary gland cells, the addition of the tricarboxylic acid-cycle substrates citrate, succinate, and pyruvate/malate restores, whereas mitochondrial uncoupling in the presence of mitochondrial substrates abolishes, the $InsP_3$ concentration-dependent modulation of Ca^{2+} oscillation frequency that is characteristic for the intact cells. Moreover, mitochondrial substrates increase the amplitudes of individual Ca^{2+} spikes and appear to facilitate Ca^{2+} oscillations at threshold $InsP_3$ concentration. This clearly indicates an important role for mitochondria in controlling $InsP_3$-mediated Ca^{2+} signalling in this system. Effects of either the activation of mitochondrial metabolism or the breakdown of the mitochondrial membrane potential on the frequency of Ca^{2+} oscillations have also been reported by others in intact cells. First, Jouaville and coworkers (1995) have shown that the microinjection of pyruvate and malate into *Xenopus* oocytes increases Ca^{2+} spike amplitudes and interspike intervals at a given $InsP_3$ concentration, whereas the blocking of mitochondrial function has the opposite effects. Subsequently, interference with mitochondrial function has also been found to affect agonist-evoked Ca^{2+} signals in oligodendrocytes and astrocytes (Simpson & Russell, 1996; Boitier *et al.* 1999). In contrast to the present study, these reports failed to clarify, however, whether the contribution of mitochondria to the control of oscillation frequency is secondary to other mechanisms or whether mitochondria provide the primary control over this process.

There are several potential mechanisms through which mitochondria might exert negative control over the Ca^{2+} oscillation frequency. First, mitochondria might control Ca^{2+} oscillation frequency through taking up Ca^{2+} ions that are released from the $InsP_3$-sensitive stores. Alternatively, addition of mitochondrial substrates or dissipation of the mitochondrial proton gradient may have produced or abolished localized gradients in [ATP] which, in turn, may have affected $InsP_3$ receptor gating (Smith *et al.* 1985; Ferris *et al.* 1990; Landolfi *et al.* 1998). However: (1) throughout the present study, glands were superfused with a high concentration of ATP (3 mM) to minimize the contributions of endogenously produced ATP and (2) addition of Ruthenium Red, an inhibitor of mitochondrial Ca^{2+} uptake, mimicked the effects of FCCP–oligomycin.

Since Ca^{2+} uptake into mitochondria via the electrogenic mitochondrial Ca^{2+} uniporter and driven by the mitochondrial membrane potential exhibits a relatively low Ca^{2+} affinity ($K_d > 1\ \mu M$), it has long been controversial whether mitochondria can play a significant role in cytoplasmic Ca^{2+} clearing at concentrations reached under physiological conditions (Carafoli, 1987; Gunter, 1994). Several recent studies, however, have demonstrated that mitochondrial Ca^{2+} uptake is indeed stimulated and can even be saturated by physiological $[Ca^{2+}]_C$ elevations (Rizzuto *et al.* 1993; Werth & Thayer, 1994; Hajnóczky *et al.* 1995; Sparagna *et al.* 1995; Rizzuto *et al.* 1998; Csordás *et al.* 1999). Thus, mitochondria positioned close to sites of Ca^{2+} influx or release are thought to be exposed to microdomains of $[Ca^{2+}]_C$ severalfold higher than the peak $[Ca^{2+}]_C$ averaged over greater regions of a cell. Energized mitochondria have therefore to be considered as stationary buffers for intracellular Ca^{2+} that may quantitatively and qualitatively influence physiological Ca^{2+} signals.

On the basis of the model for Ca^{2+} oscillations outlined above, a regenerative Ca^{2+} spike will be triggered when $InsP_3$-mediated Ca^{2+} efflux via $InsP_3$ receptors causes $[Ca^{2+}]_C$ to exceed the threshold for the activation of neighbouring closed, but excitable $InsP_3$ receptors, i.e. when $InsP_3$-induced Ca^{2+} efflux from $InsP_3$-sensitive Ca^{2+} stores critically exceeds the rate of Ca^{2+} removal by all Ca^{2+}-buffering mechanisms. Ca^{2+} uptake into energized mitochondria positioned close to the sites of release would thus retard $InsP_3$-induced $[Ca^{2+}]_C$ rises, thereby delaying Ca^{2+} mediated activation of closed $InsP_3$ receptors and increasing interspike intervals at constant $[InsP_3]$ (Jouaville *et al.* 1995; and present study). Such an effect of mitochondrial Ca^{2+} uptake can directly explain the $InsP_3$ concentration-dependent modulation of Ca^{2+} oscillations in the presence of mitochondrial substrates, as shown in this study, because increasing $InsP_3$-mediated Ca^{2+} release will increase the rate of $[Ca^{2+}]_C$ rise and consequently reduce the time required to generate the Ca^{2+} trigger necessary for eliciting a Ca^{2+} spike. Conversely, in the absence of mitochondrial Ca^{2+} uptake, rates of $[Ca^{2+}]_C$ rise may become so fast that they, even at low $[InsP_3]$, no longer limit oscillation frequency. Under these conditions, the recovery of $InsP_3$ receptors from Ca^{2+}-dependent inactivation may become a rate-limiting step, resulting in the high-frequency Ca^{2+} oscillations with all-or-none characteristics observed in the absence of mitochondrial substrates and after mitochondrial uncoupling in the presence of substrates. In addition to delaying positive Ca^{2+} feedback, mitochondrial Ca^{2+} uptake may also delay negative Ca^{2+} feedback operative at higher $[Ca^{2+}]_C$. This would prolong the opening of $InsP_3$ receptor channels and result in enhanced transients of $[Ca^{2+}]_C$ and $[Ca^{2+}]_L$ as observed in the permeabilized blowfly salivary gland and intact *Xenopus* oocytes (Jouaville *et al.* 1995).

In summary, the data presented in this study emphasize the role of mitochondria in controlling the amplitude of Ca^{2+} spikes and the duration of interspike intervals in $InsP_3$-mediated Ca^{2+} oscillations. They indicate that energized mitochondria are essential for the generation of temporal patterns of Ca^{2+} oscillations that are related to the concentration of $InsP_3$ or agonist. Furthermore, they raise the possibility that *in vivo* changes in mitochondrial membrane potential, either as a consequence of changes in the cell's metabolic state or because of active regulation, may modulate the spatio-temporal organization of $InsP_3$-induced intracellular Ca^{2+} signals.

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