J Physiol 580.1 (2007) pp 39–49

Modulation of Ca^{2+} release and Ca^{2+} oscillations in HeLa cells and fibroblasts by mitochondrial Ca^{2+} uniporter stimulation

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The recent availability of activators of the mitochondrial Ca^{2+} uniporter allows direct testing of the influence of mitochondrial Ca^{2+} uptake on the overall Ca^{2+} homeostasis of the cell. We show here that activation of mitochondrial Ca^{2+} uptake by 4,4′,4″-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT) or kaempferol stimulates histamine-induced Ca^{2+} release from the endoplasmic reticulum (ER) and that this effect is enhanced if the mitochondrial Na^+–Ca^{2+} exchanger is simultaneously inhibited with CGP37157. This suggests that both Ca^{2+} uptake and release from mitochondria control the ability of local Ca^{2+} microdomains to produce feedback inhibition of inositol 1,4,5-trisphosphate receptors ($InsP_3Rs$). In addition, the ability of mitochondria to control Ca^{2+} release from the ER allows them to modulate cytosolic Ca^{2+} oscillations. In histamine stimulated HeLa cells and human fibroblasts, both PPT and kaempferol initially stimulated and later inhibited oscillations, although kaempferol usually induced a more prolonged period of stimulation. Both compounds were also able to induce the generation of Ca^{2+} oscillations in previously silent fibroblasts. Our data suggest that cytosolic Ca^{2+} oscillations are exquisitely sensitive to the rates of mitochondrial Ca^{2+} uptake and release, which precisely control the size of the local Ca^{2+} microdomains around $InsP_3Rs$ and thus the ability to produce feedback activation or inhibition of Ca^{2+} release.

(Resubmitted 11 December 2006; accepted after revision 15 January 2007; first published online 18 January 2007)

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Over the last decade, evidence has been growing regarding the participation of mitochondria in the control of global cellular Ca^{2+} homeostasis. The mitochondrial Ca^{2+} uptake mechanism has both high rate and low Ca^{2+} affinity and appears to be specially designed to take up Ca^{2+} from local microdomains of high $[Ca^{2+}]$, thus controlling their size and magnitude. High Ca^{2+} microdomains are usually generated in close proximity to open Ca^{2+} channels, either on the cytosolic side of the plasma membrane (for plasma membrane Ca^{2+} channels) or on the cytosolic side of the endoplasmic reticulum (ER) (e.g. for inositol 1,4,5-trisphosphate ($InsP_3$) receptors ($InsP_3Rs$)). Mitochondria placed close to these channels may take up large amounts of Ca^{2+} and thus modulate the amplitude of the microdomain and its physiological function. In fact, we have shown in chromaffin cells that mitochondria are able to take up transiently most of the Ca^{2+} entering the cells through Ca^{2+} channels during cell stimulation (Villalobos *et al.* 2002). Thus, acting as transient Ca^{2+} buffers, mitochondria can modulate physiological phenomena triggered by cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_c$), such as secretion (Giovannucci *et al.* 1999; Montero *et al.* 2000).

In non-excitable cells, regenerative Ca^{2+} oscillations and waves can be produced by several mechanisms (for reviews see Putney & Bird, 1993; Fewtrell, 1993; Berridge & Dupont, 1994; Miyakawa *et al.* 2001; Hattori *et al.* 2004), but a key element is the dual positive and negative feedback regulation of $InsP_3Rs$ by the released Ca^{2+}. Opening of $InsP_3Rs$ requires both $InsP_3$ and Ca^{2+} in the submicromolar range but an increase in the local $[Ca^{2+}]_c$ above the micromolar range becomes inhibitory (Bezprozvanny *et al.* 1991; Kaftan *et al.* 1997). Thus, mitochondria placed close to $InsP_3Rs$ in the ER may be able to control their activity by modulating the $[Ca^{2+}]_c$ microenvironment in the cytosolic mouth of the channel. In fact, there is both structural and functional evidence suggesting the presence of specific and stable interactions between mitochondria and ER which facilitate a rapid and nearly

DOI: 10.1113/jphysiol.2006.126391

direct flux of Ca^{2+} from ER to mitochondria (Rizzuto *et al.* 1998; Hajnoczky *et al.* 1999, 2000; Filippin *et al.* 2003). These tight ER–mitochondria couplings may also serve to modulate Ca^{2+} release.

The role of mitochondria in cytosolic Ca^{2+} signalling has been tested mostly by using protonophores or respiratory chain inhibitors to depolarize the mitochondrial membrane, thus abolishing the driving force for Ca^{2+} uptake into the organelle. Usually, the $[Ca^{2+}]_c$ transient induced by different stimuli is larger when mitochondria are depolarized, confirming that mitochondria take up significant amounts of Ca^{2+} during cell stimulation (Werth & Thayer, 1994; White & Reynolds, 1997; Babcock *et al.* 1997; Montero *et al.* 2001). In addition, mitochondrial depolarization inhibits the production of regenerative oscillations (Collins *et al.* 2000) and facilitates ER Ca^{2+} depletion (Arnaudeau *et al.* 2001; Malli *et al.* 2003) in histamine-stimulated HeLa cells. On the other hand, we have shown recently that inhibition with CGP37157 of Ca^{2+} efflux from mitochondria through the mitochondrial Na^+–Ca^{2+} exchanger (MNCE) changes the pattern of oscillations in HeLa cells and produces regenerative oscillations in human fibroblasts (Hernández-SanMiguel *et al.* 2006). CGP37157 also activated Ca^{2+} release from the ER (Hernández-SanMiguel *et al.* 2006) and reduced ER Ca^{2+} refilling (Arnaudeau *et al.* 2001; Malli *et al.* 2005). Thus, MNCE has been implicated in the control of ER Ca^{2+} release and Ca^{2+} oscillations (Hernández-SanMiguel *et al.* 2006), ER–mitochondria Ca^{2+} recycling (Arnaudeau *et al.* 2001) and the transfer of Ca^{2+} from the extracellular medium to the ER through mitochondria (Malli *et al.* 2003, 2005).

We have taken advantage here of the recent availability of strong activators of the mitochondrial Ca^{2+} uniporter (MCU; see Montero *et al.* 2002, 2004; Lobatón *et al.* 2005) to investigate the role of mitochondrial Ca^{2+} uptake in the control of ER Ca^{2+} release and cytosolic Ca^{2+} oscillations. We show here that these phenomena are highly sensitive to changes in the activity of the MCU, thus providing new evidence for the critical role of mitochondria in the control of global cell Ca^{2+} homeostasis.

Methods

Cell culture and targeted aequorin expression

HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. The constructs for aequorin targeted to the cytosol and mutated aequorin targeted to either the ER or the mitochondria have been previously described (Montero *et al.* 1995, 2000). Transfections were carried out using Metafectene (Biontex, Munich, Germany). Cultures of human fibroblasts were obtained from skin biopsies of healthy human volunteers. They were grown in 199 medium supplemented with 10% fetal calf serum.

Mitochondrial and ER [Ca^{2+}] measurements in cell populations with targeted aequorin

Mitochondrial [Ca^{2+}] ($[Ca^{2+}]_m$) measurements were made using wild-type HeLa cells transfected with the pcDNA3.1 plasmid containing the construct for mitochondrially targeted mutated aequorin. For aequorin reconstitution, HeLa cells expressing mitochondrially targeted mutated aequorin were incubated for 1–2 h at room temperature (20°C) with 1 μM wild-type coelenterazine in standard medium containing (mM): NaCl 145, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1, glucose 10 and Hepes 10; pH 7.4. Cells were then placed in the perfusion chamber of a purpose-built luminometer thermostatically controlled at 37°C. ER [Ca^{2+}] ($[Ca^{2+}]_{ER}$) measurements were carried out using HeLa cells transiently transfected with the plasmid for ER-targeted aequorin. Cells were plated onto 13 mm round coverslips. Before reconstituting aequorin, $[Ca^{2+}]_{ER}$ was reduced by incubating the cells for 10 min at 37°C with the sarcoplasmic reticulum and ER Ca^{2+}-ATPase inhibitor 2,5-di-tert-buthyl-benzohydroquinone (BHQ; 10 μM) in medium containing (mM): NaCl 145, KCl 5, $MgCl_2$ 1, glucose 10 and Hepes 10; pH 7.4, supplemented with 0.5 mM EGTA. Cells were then washed and incubated for 1 h at room temperature in the same medium with 1 μM coelenterazine n, a low sensitivity analog of wild type coelenterazine which allows measuring the higher [Ca^{2+}] present in the ER. Then, the coverslip was placed in the perfusion chamber of a purpose-built thermostatically controlled luminometer, and the same medium containing 0.5 mM EGTA was perfused for 5 min prior to the experiment.

Single-cell $[Ca^{2+}]_c$ measurements

HeLa cells or fibroblasts were loaded with fura-2 by incubation in standard medium containing 2 μM acetoxymethyl ester form of fura-2- (fura-2-AM) for 45 min at room temperature. Cells were then washed with standard medium for 45 min at room temperature and mounted in a cell chamber on the stage of a Zeiss Axiovert 200 microscope under continuous perfusion. Single-cell fluorescence was excited at 340 nm and 380 nm using a Cairn monochromator (100 ms excitation at each wavelength every 2 s, 10 nm bandwidth) and images of the emitted fluorescence obtained with a 40 × Fluar objective were collected using a 400DCLP dichroic mirror and a D510/80 emission filter (both from Chroma Technology) and recorded with a Hamamatsu ORCA-ER camera. Single-cell fluorescence was recorded as 340/380 nm fluorescence ratio and calibrated into [Ca^{2+}] values off-line as previously described (Grynkiewicz *et al.* 1985) using the Metafluor program (Universal Imaging). Experiments were performed at 37°C using an on-line heater from Harvard Apparatus.

Materials

Wild-type coelenterazine, coelenterazine n and fura-2-AM were obtained from Molecular Probes, OR, USA. CGP37157, PPT and kaempferol were from Tocris, Bristol, UK. Other reagents were from Sigma, Madrid or Merck, Darmstadt.

Results

We have shown before that the synthetic oestrogen agonist PPT is a potent activator of Ca^{2+} uptake into mitochondria both in intact and permeabilized cells (Lobatón *et al.* 2005). PPT largely increased (up to 6-fold) the [Ca^{2+}]$_m$ peak induced by histamine in HeLa cells (Lobatón *et al.* 2005), an effect that was not secondary to an increased Ca^{2+} release from the ER, as the [Ca^{2+}]$_c$ peak induced by histamine was in fact slightly reduced in the presence of PPT (by about 20%). However, we did not explore further the effects of PPT on ER Ca^{2+} release and [Ca^{2+}]$_c$ dynamics. We have recently described that inhibiting mitochondrial Ca^{2+} release with CGP37157 activates Ca^{2+} release from the ER (Hernández-SanMiguel *et al.* 2006) and promotes the production of regenerative cytosolic Ca^{2+} oscillations in HeLa cells and fibroblasts. Figure 1*A* shows that activation of the MCU with PPT also enhanced histamine-induced Ca^{2+} release form the ER and that the effect was dose-dependent within the same range of concentrations required to activate the MCU. In addition, Fig. 1*B* shows that CGP37157 potentiated the activation of ER Ca^{2+} release induced by PPT, suggesting that both activation of mitochondrial Ca^{2+} uptake and inhibition of mitochondrial Ca^{2+} release cooperate to activate Ca^{2+} release from the ER.

As we have reported previously (Montero *et al.* 1997; see Fig. 1), Ca^{2+} release induced by histamine in these cells is biphasic. It starts with a very fast initial drop of [Ca^{2+}]$_{ER}$ lasting for about 10 s that suddenly stops and is followed by a slower phase of release which continues as long as histamine is present. The first phase is responsible for the peak of [Ca^{2+}]$_c$ and the second one keeps [Ca^{2+}]$_c$ in at an elevated level while histamine is present. It is worth mentioning here that the large increase in mitochondrial Ca^{2+} uptake induced by PPT led to a decrease in the histamine-induced [Ca^{2+}]$_c$ peak (Lobatón *et al.* 2005), in spite of the fact that PPT enhanced both phases of Ca^{2+} release. In a series of experiments similar to those shown in Fig. 1, the percentage decrease in [Ca^{2+}]$_{ER}$ during the fast phase was (mean ± s.e.m.): controls, 17.2 ± 1.2% ($n = 16$); 2 μM PPT, 25.3 ± 1.5% ($n = 16$); 5 μM PPT, 34.5 ± 2.5% ($n = 7$); 2 μM PPT + 10 μM CGP37157, 33.7 ± 2.8% ($n = 7$) and the total Ca^{2+} released in both phases, measured 3 min after histamine addition, was (mean ± s.e.m.): controls, 40.3 ± 2.8% ($n = 16$); 2 μM PPT, 59.8 ± 2.6% ($n = 16$); 5 μM PPT, 75.5 ± 2.5% ($n = 7$); 2 μM PPT + 10 μM CGP37157, 71.6 ± 3.3% ($n = 7$). Therefore, in the presence of 2 μM PPT, the amount of Ca^{2+} released by the ER in response to histamine increased by about 50% in both phases, and in the

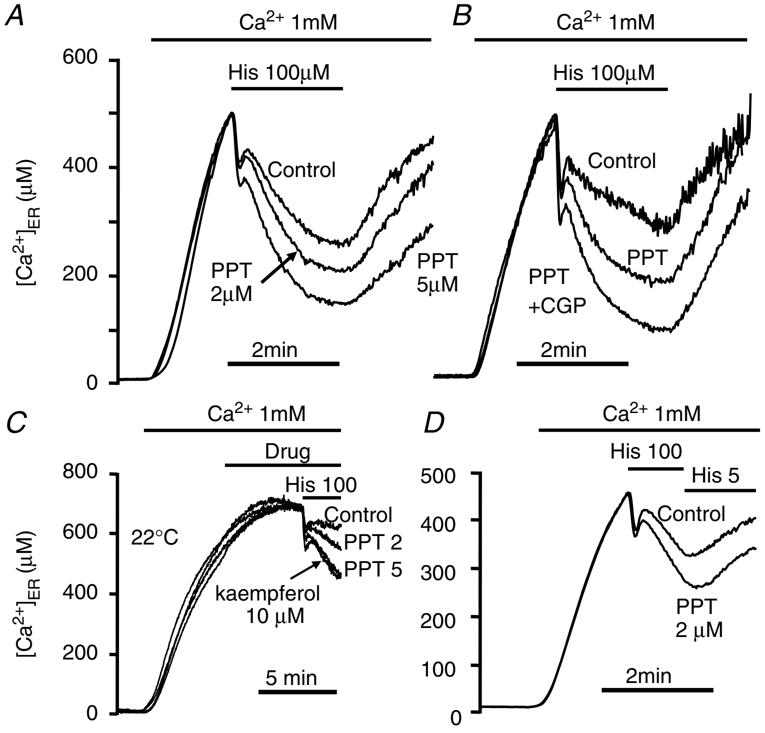

Figure 1. Effects of 4,4′,4′′-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT), kaempferol and CGP37157 on histamine-induced Ca^{2+} release from the ER

HeLa cells expressing endoplasmic reticulum (ER)-targeted mutated aequorin were reconstituted with coelenterazine n in 0.5 mM EGTA-containing medium as described in the Methods. As indicated, 1 mM Ca^{2+}-containing standard medium was perfused to refill the ER with Ca^{2+}. Cells were then stimulated with histamine (100 μM or 5 μM) either under control conditions or in the presence of PPT, CGP37157 or kaempferol at the concentrations indicated (micromolar). These compounds were also present in the Ca^{2+}-containing medium used to refill the ER in *A*, *B* and *D*, and were added when indicated (drug) in *C*. In *B*, drug concentrations were: PPT, 2 μM; CGP37157, 10 μM. Experiments shown in *C* were performed at 22°C, and the rest at 37°C. In *A* and *B*, the traces shown are the mean of two experiments of each type and they are representative of 10–12 similar experiments of each type. The traces shown in *C* are the mean of three experiments of each kind. The traces shown in *D* are each the mean of 15 experiments.

presence of 5 μM PPT it increased almost 2-fold. Given that the $[Ca^{2+}]_c$ peak obtained was smaller than in the control, this implies that all the additional Ca^{2+} released in the presence of the MCU activator was taken up by mitochondria. In the experiments shown in Fig. 1*A* and *B*, the agonist was added before the ER was fully refilled with Ca^{2+}. The reason for adding histamine so early is the fast consumption of aequorin at the high $[Ca^{2+}]$ reached in the ER, which means that $[Ca^{2+}]_{ER}$ can only be measured for a few minutes (Alvarez & Montero, 2002). To be sure that the effects of PPT also occurred when $[Ca^{2+}]_{ER}$ was at steady state, we performed similar experiments at a lower temperature. We have previously described that reducing the temperature to 22°C reduces the rate of light emission of aequorin, so that longer records of $[Ca^{2+}]_{ER}$ can be obtained (Barrero *et al.* 1997; Alvarez & Montero, 2002). Figure 1*C* shows that PPT also produced an increase in the rate of Ca^{2+} release from the ER under steady-state $[Ca^{2+}]_{ER}$. This figure also shows that the flavonoid kaempferol, which is also a potent activator of the mitochondrial Ca^{2+} uniporter (Montero *et al.* 2004) but has a completely different chemical structure, similarly activates Ca^{2+} release from the ER. In addition, this figure shows that application of PPT or kaempferol alone produces no change in $[Ca^{2+}]_{ER}$.

If PPT activates $InsP_3$-induced Ca^{2+} release, we reasoned that it could also modify the dynamics of cytosolic Ca^{2+} oscillations, as occurs with CGP37157 (Hernández-SanMiguel *et al.* 2006). That was the case, although the effect of PPT was different from that of CGP37157. In the single-cell experiments, we stimulated HeLa cells initially with 100 μM histamine and then a lower histamine concentration (3–5 μM) was maintained in order to reduce the frequency and facilitate the generation of long-lasting oscillations. Figure 1*D* shows the effect of this protocol on Ca^{2+} release from the ER. When histamine was reduced from 100 to 5 μM, $[Ca^{2+}]_{ER}$ increased both in the presence and in the absence of PPT, but remained lower in the presence of PPT compared with the controls. Figure 2*A* shows that in HeLa cells, histamine-induced Ca^{2+} oscillations progressively decreased in frequency and

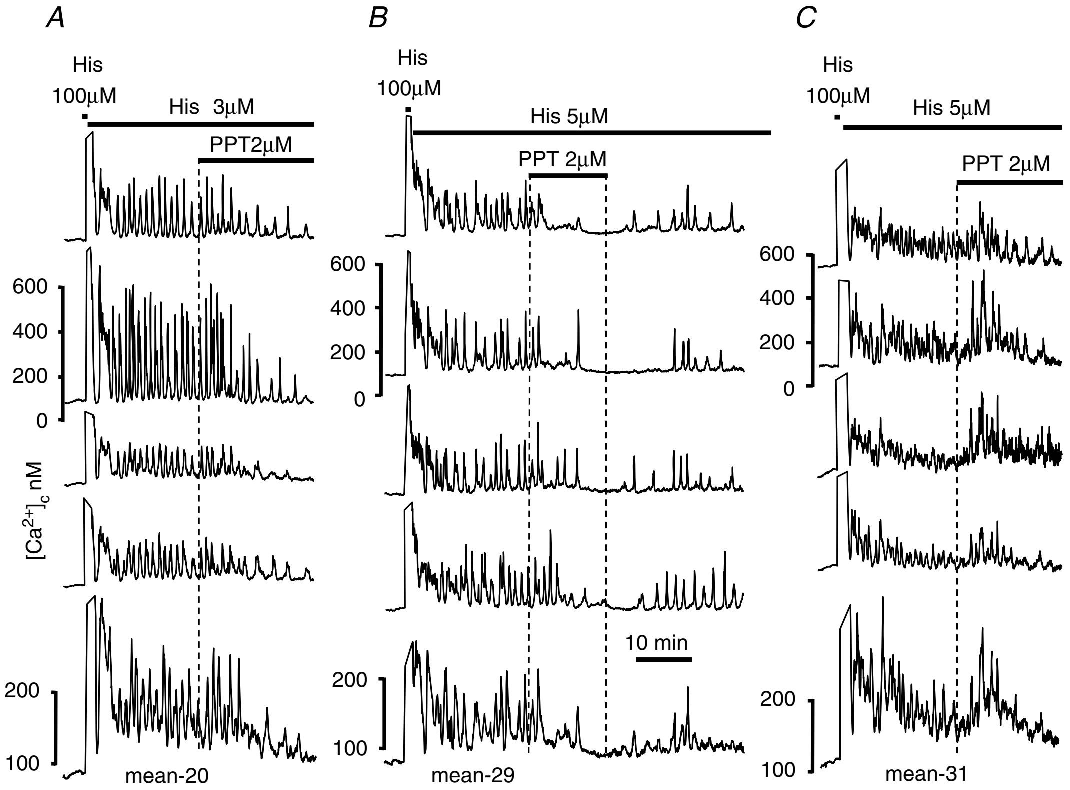

Figure 2. Effect of 4,4′,4″-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT) on histamine-induced Ca^{2+} oscillations

Fura-2-loaded HeLa cells incubated in 1 mM Ca^{2+}-containing standard medium were stimulated with histamine and treated with PPT as indicated. *A*–*C*, show single-cell Ca^{2+} records from three different experiments. Traces of four representative single cells present in the same microscope field for each experiment are shown. The bottom traces show the mean of all the cells analysed in each experiment (20, 29 and 31 cells for *A*, *B* and *C*, respectively; note the enhanced scale). The initial histamine-stimulated $[Ca^{2+}]_c$ peaks are truncated for convenience. Data are representative of 412 analysed cells.

amplitude after perfusion of PPT and finally stopped. This behaviour was observed in 87% of the cells (358 of 412 analysed cells), while either no effect or an increase in frequency was observed in the rest. Reversion of this effect was very slow and was observed only in some cells (17%, 40 of 234 cells in which recovery was measured). Figure 2*B* shows data from an experiment in which oscillations reappeared in several cells about 10 min after PPT was washed out. It is interesting to note that in many cells, the blocking effect of PPT was preceded by a transient increase in the magnitude or width of the oscillations, suggesting that an increase in Ca^{2+} release was the primary effect of PPT. In fact, in experiments where cells had low-amplitude or irregular oscillations, PPT addition generated a transient burst of oscillations (Fig. 2*C*).

Similar findings were obtained in histamine-stimulated HeLa cells treated with CGP37157 to induce the generation of baseline spike oscillations. Figure 3 shows that, as previously shown (Hernández-SanMiguel *et al.* 2006), addition of CGP37157 changed the pattern of the Ca^{2+} oscillations, particularly in those cells showing a more irregular pattern beforehand. Then, subsequent perfusion of PPT decreased again both frequency and amplitude of the oscillations, leading to a complete cessation after 5–10 min. This behaviour was observed in 95% of the cells (307 of 322 analysed cells). Again here, PPT induced in some cells a burst of oscillations before blocking them (see second trace from top and also the bottom trace, which shows the mean response of the 29 cells present in the same microscope field). Reversion of the blocking effect of PPT (as shown in Fig. 3) was observed only in some cells (12%, 31 of 261 cells in which recovery was assayed) and was also quite slow, requiring a washout period of at least 10–20 min. The reason for this slow recovery of the oscillatory behaviour is probably the slow reversion of the effect of PPT. Table 1 shows the rate of disappearance of the effect of PPT on the histamine-induced $[Ca^{2+}]_m$ peak. In the presence of 2 μM PPT, the $[Ca^{2+}]_m$ peak increased about 3-fold over the control values. Washing out PPT then reduced its effect slowly, so that after 10 min washout, the $[Ca^{2+}]_m$ peak was still about 50% higher than in the controls. Similar findings were originally reported for SB202190 (Montero *et al.* 2002).

Table 1. Rate of reversal of the effect of 4,4′,4′′-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT) on the histamine-induced mitochondrial $[Ca^{2+}]$ ($[Ca^{2+}]_m$) peak

	Condition	Histamine-induced $[Ca^{2+}]_m$ peak (μM)
(a)	With 2 μm PPT present	69 ± 4
(b)	2 min after PPT washout	52 ± 3
(c)	4 min after PPT washout	43 ± 2
(d)	10 min after PPT washout	35 ± 3
(e)	Control cells	23 ± 2

HeLa cells expressing mitochondrially targeted mutated aequorin were reconstituted with native coelenterazine and stimulated for 1 min with 100 μM histamine either in control cells (e) or in the following conditions: cells incubated with 2 μM PPT for 5 min and then treated with histamine in the presence of PPT (a), or cells incubated with 2 μM PPT for 5 min, then washed with 1 mM Ca^{2+}-containing standard medium for 2 (b), 4 (c) or 10 (d) min and then treated with histamine. Data are means ± S.E.M., $n = 10$.

To obtain further evidence that the effects of PPT were due to stimulation of MCU, we have also studied the effects of kaempferol on Ca^{2+} oscillations. As mentioned above, this compound is a flavonoid with a chemical structure completely different from that of PPT, but it is also a potent activator of MCU (Montero *et al.* 2004). We showed in Fig. 1*C* that it produced the same effects as PPT on histamine-induced Ca^{2+} release from the ER. Now we show in Fig. 4 its effects on Ca^{2+} oscillations. We have used a concentration (10 μM) that produces an activation of MCU similar to that induced by 2 μM PPT. As we have shown before (Montero *et al.* 2004; Lobatón *et al.* 2005), both 2 μM PPT and 7 μM kaempferol increased by 10-fold the rate of Ca^{2+} uptake by mitochondria. Addition of

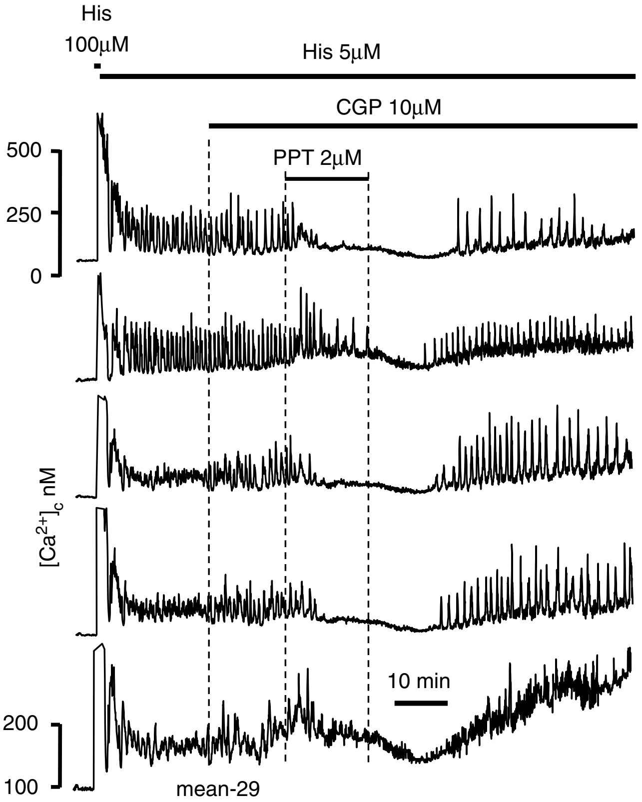

Figure 3. Effect of 4,4′,4′′-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT) on histamine-induced Ca^{2+} oscillations in the presence of CGP37157

Fura-2-loaded HeLa cells incubated in 1 mM Ca^{2+}-containing standard medium were stimulated with histamine and treated with 10 μM CGP37157 and 2 μM PPT as indicated. The bottom trace shows the mean of all the cells analysed in the experiment (29 cells). Data are representative of 322 analysed cells.

kaempferol always produced an initial burst of activity, similar to that induced by PPT although usually more prolonged. That initial burst was followed by a series of oscillations with progressively smaller amplitude and in most cases the oscillatory behaviour final ceased. Figure 4*A* shows two typical experiments in which kaempferol induced a more or less prolonged burst of oscillations. The bottom traces, which correspond to the mean behaviour of all the cells present in the same microscope field, show that most of the cells responded in the same way.

We next investigated the behaviour of $[Ca^{2+}]_m$ in the presence of cytostolic Ca^{2+} oscillations and one or more of these compounds (experiments similar to those shown in Figs 2 and 3). Figure 5 shows the effects of PPT, kaempferol and CGP37157 on $[Ca^{2+}]_m$ under similar conditions to those used in Figs 2–4. Measurements were performed in cell populations expressing mitochondrially targeted mutated aequorin (Montero *et al.* 2002). HeLa cells were stimulated with histamine, and then either PPT, kaempferol, CGP37157 or the combination of CGP37157 and one of the two MCU activators was applied. As we have previously described, inhibition of MNCE with CGP37157 produced a small and slow increase in the mean $[Ca^{2+}]_m$ within the submicromolar range (Hernández-SanMiguel *et al.* 2006). Instead, stimulation of the MCU with PPT did not produce by itself any significant change in the mean $[Ca^{2+}]_m$, but strongly potentiated the effect of CGP37157. In the case of kaempferol, it produced a small increase in $[Ca^{2+}]_m$ by itself, which was also enhanced by CGP37157. The mean increase in $[Ca^{2+}]_m$ observed 5 min after the addition of each drug in experiments similar to those of Fig. 5 was (mean ± s.e.m.): 2 μM PPT alone, 0.02 ± 0.01 μM ($n = 22$); 10 μM kaempferol alone, 0.28 ± 0.03 ($n = 16$); 10 μM CGP alone, 0.20 ± 0.04 μM ($n = 25$); 2 μM PPT + 10 μM CGP37157, 0.79 ± 0.13 μM ($n = 26$); 10 μM kaempferol + 10 μM CGP37157, 0.54 ± 0.03 ($n = 16$).

We then investigated the effect of stimulating MCU on Ca^{2+} oscillations in human fibroblasts. Figure 6 shows that in human fibroblasts stimulated with CGP37157 to produce oscillations, PPT induced similar effects to those observed in HeLa cells; that is, a decrease in frequency or cessation of the oscillatory behaviour. This kind of effect was observed in most (95%, 61 of 64 analysed cells) of the cells exposed to this experimental protocol. Reversion of this PPT block, which was only observed in some of the cells (41%, 11 of 27 cells in which recovery was assayed), again required a prolonged (∼10 min) washout period for PPT. However, in the absence of CGP37157, the response

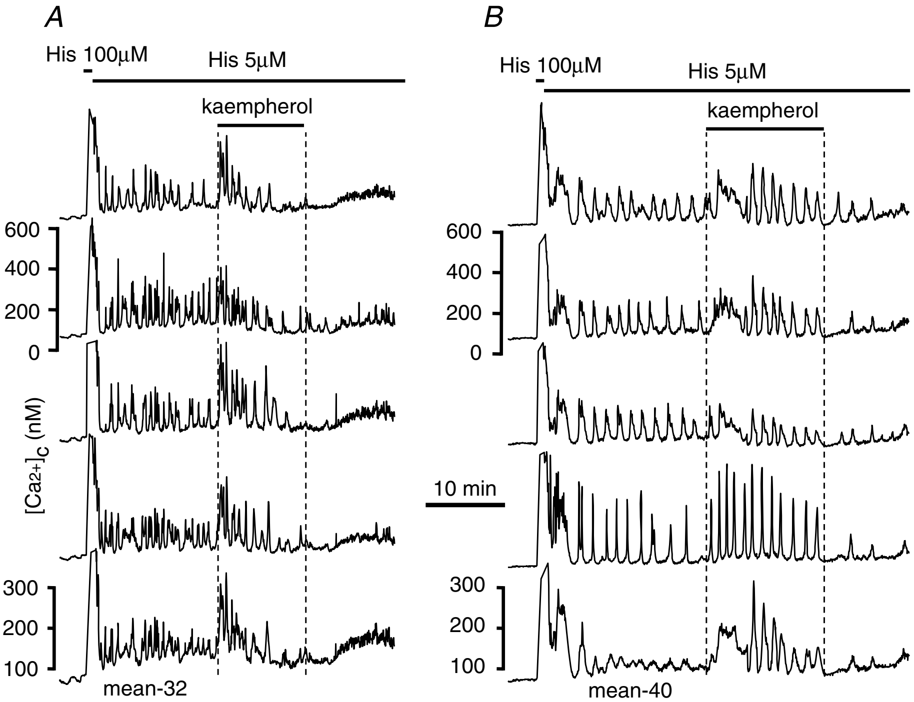

Figure 4. Effect of kaempferol on histamine-induced Ca^{2+} oscillations

Fura-2-loaded HeLa cells incubated in 1 mM Ca^{2+}-containing standard medium were stimulated with histamine and treated with 10 μM kaempferol as indicated. Traces of four representative single cells present in the same microscope field for each experiment are shown. The bottom traces show the mean of all the cells analysed in each experiment (32 and 40 cells for *A* and *B*, respectively). Data are representative of 352 cells treated with the same protocol.

of $[Ca^{2+}]_c$ dynamics to PPT perfusion was more diverse. In about half of the cells (52%, 121 of 231 analysed cells), the response was again similar to that observed in HeLa cells; that is, an initial stimulation followed by inhibition of the oscillations. Figure 7*A* shows an experiment in which PPT stopped the spontaneous oscillations almost completely within a few minutes, an effect that was usually not reversible. In other cells, instead, PPT increased the frequency of the oscillations (11%, 25 of 231 analysed cells, see Fig. 7*B*), induced the generation of oscillations in cells that were previously silent (28%, 65 of 231 analysed cells, see Fig. 7*C*) or had no effect (9%, 20 of 231 analysed cells). Stimulation or generation of Ca^{2+} oscillations was even more frequent when kaempferol was used to stimulate MCU. This flavonoid increased the frequency or amplitude of the oscillations in all the cells tested having spontaneous oscillations (100%, 32 of 32) and induced the generation of oscillations in about half of the cells (47%, 49 of 105 analysed cells) that were silent under resting conditions. Figure 8*A* shows single-cell traces representative of these two behaviours. However, in the presence of CGP37157, kaempferol predominantly inhibited oscillations after an initial burst of activity (51%, 26 of 51 analysed cells), although either no effect (20%, 10 of 51 analysed cells) or a persistent stimulation of the oscillatory behaviour (29%, 15 of 51 analysed cells) was also observed. Figure 8*B* shows representative single-cell traces.

Discussion

We show in this paper new evidence that mitochondrial Ca^{2+} uptake modulates $[Ca^{2+}]$ dynamics in the cytosol. We have used two activators of MCU, recently described by us, to show that the rate of Ca^{2+} uptake by mitochondria controls Ca^{2+} release from the ER and cytosolic Ca^{2+} oscillations. The two compounds, the synthetic oestrogen receptor agonist PPT and the flavonoid kaempferol, have completely different molecular structures, but they similarly activate MCU (Montero *et al.* 2004; Lobatón *et al.* 2005) and we show here that they also both activate Ca^{2+} release from the ER. Regarding Ca^{2+} oscillations, the modulation appears to be quite subtle – a sort of fine tuning – as activation of MCU may trigger both activation and inhibition of the oscillatory behaviour, even in the same type of cells. In HeLa cells, where Ca^{2+} oscillations are induced by histamine, activation of MCU produced in most of the cells an initial stimulation followed by inhibition of the oscillations. This effect developed slowly,

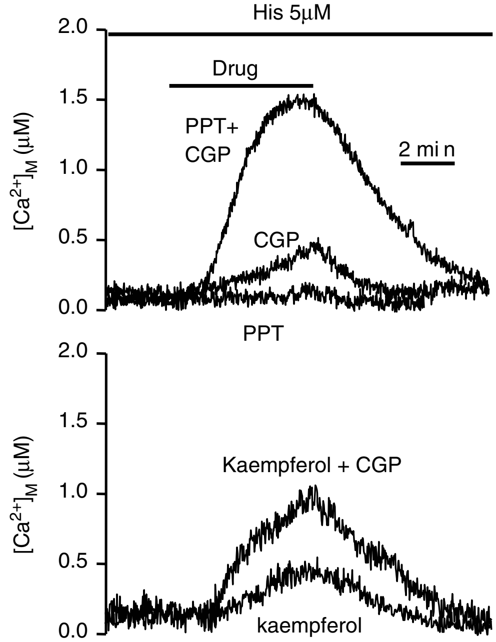

Figure 5. Effect of 4,4′,4′′-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT), CGP37157, kaempferol, and the combinations of PPT + CGP37157 and kaempferol + CGP37157 on $[Ca^{2+}]_m$ in histamine-stimulated HeLa cells
HeLa cells expressing mitochondrially targeted mutated aequorin were reconstituted with native coelenterazine, stimulated for 1 min with 100 µM histamine and then perfused with medium containing 5 µM histamine and either 2 µM PPT, 10 µM CGP37157, 10 µM kaempferol or the drug combinations as indicated. All perfusion media contained 1 mM Ca^{2+}. Mean values for the effect of each drug on $[Ca^{2+}]_m$ are given in the text.

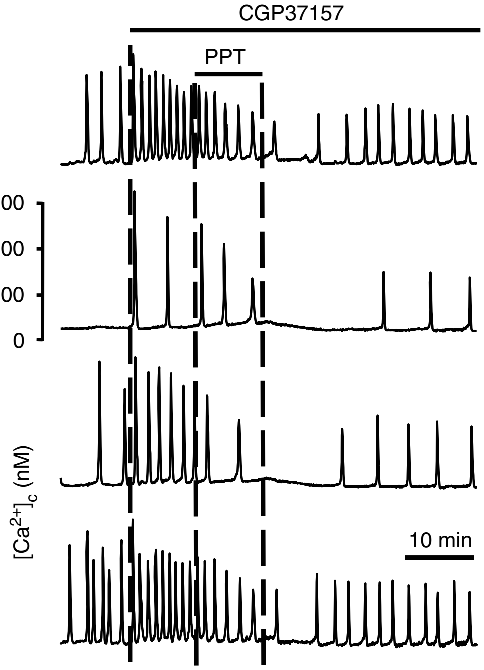

Figure 6. Effect of 4,4′,4′′-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT) on Ca^{2+} oscillations stimulated with CGP37157 in human fibroblasts
Fura-2-loaded human fibroblasts incubated in 1 mM Ca^{2+}-containing medium were treated with 10 µM CGP37157 and 2 µM PPT as indicated. Traces correspond to four cells present in the same microscope field in one experiment (of a total of six cells analysed).

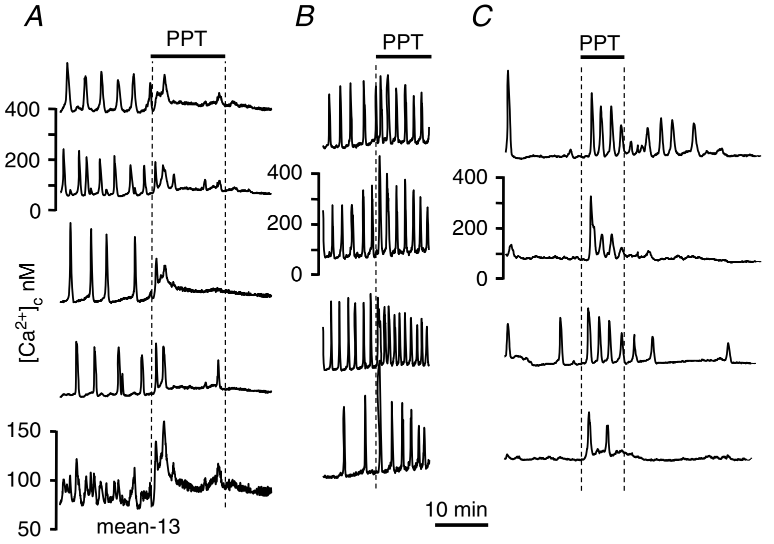

Figure 7. Effect of 4,4′,4′′-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT) on spontaneous Ca^{2+} oscillations in human fibroblasts

Fura-2-loaded human fibroblasts incubated in 1 mM Ca^{2+}-containing medium were treated with 2 μM PPT as indicated. *A–C*, show data from three different experiments. The bottom trace in *A* shows the mean of all the cells analysed in that experiment (13 cells).

within 2–5 min of perfusion of the activator, and was also slowly reversible. Both MCU activators (PPT and kaempferol) behaved similarly, although the initial period of stimulation was more prolonged in the presence of kaempferol.

In human fibroblasts, cells undergoing spontaneous Ca^{2+} oscillations and silent cells coexist under resting conditions. In these cells, the effects of PPT and kaempferol were more diverse. In many of the silent cells, PPT and particularly kaempferol induced the generation of Ca^{2+} oscillations. However, in cells showing spontaneous oscillations, both compounds behaved differently. PPT abolished or reduced the frequency of the oscillations in most of them, although in a small number (11%) the opposite effect was seen: an increase in the frequency of the oscillations. Instead, kaempferol increased the frequency of the oscillations in all the cells tested. On the other hand, in cells stimulated to oscillate with CGP37157, both PPT and kaempferol inhibited oscillations in most of them, although kaempferol was again able to induce a prolonged stimulation in some cells. In summary, both compounds stimulate Ca^{2+} release from the ER and produce an initial increase of the oscillatory activity. The duration of such increased activity apparently depends on the compound used (more prolonged stimulation with kaempferol) or on the previous activity of the cell (more prolonged stimulation in cells previously silent compared with active cells or cells stimulated to oscillate with CGP37157).

The reason for the different effect of MCU activation in active or silent fibroblasts may be due to the fact that excess activation of Ca^{2+} release may lead to ER Ca^{2+} depletion and feedback inhibition of Ca^{2+} release induced by the ER Ca^{2+} depletion. It is known that $InsP_3Rs$ are regulated by the level of luminal $[Ca^{2+}]$ (Camacho & Lechleiter, 1995; Caroppo *et al.* 2003; Higo *et al.* 2005) and depletion of $[Ca^{2+}]_{ER}$ below certain levels may lead to a prolonged

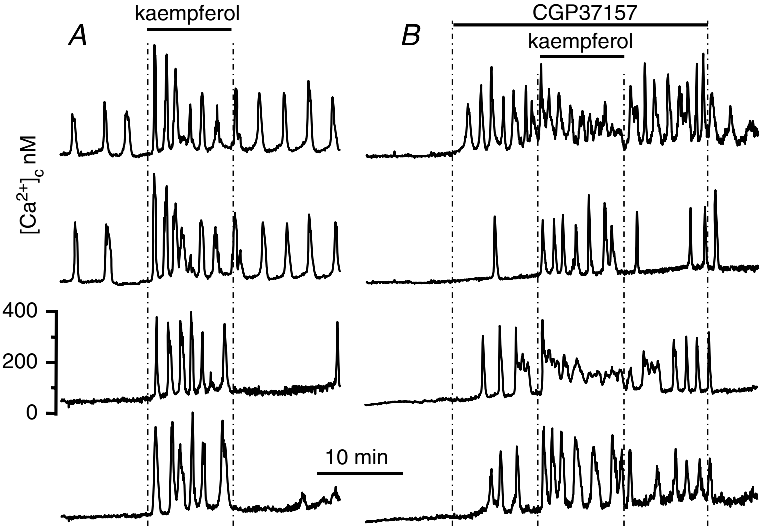

Figure 8. Effect of kaempferol on spontaneous and CGP37157-induced Ca^{2+} oscillations in human fibroblasts

Fura-2-loaded human fibroblasts incubated in 1 mM Ca^{2+}-containing medium were treated with 10 μM CGP37157 and/or 10 μM kaempferol, as indicated. Representative single-cell traces for each protocol are shown.

inhibition of the oscillatory activity. Most of the cells in which MCU activation abolished oscillations showed a short burst of activity beforehand (see Figs 2–4 and 6–8). By contrast, in silent cells, the activation of Ca^{2+} release induced by MCU activators may be just enough to induce them to oscillate.

The mechanism of the effects of MCU activation on Ca^{2+} release is probably related to the regulation of $InsP_3Rs$ by the local $[Ca^{2+}]$ surrounding the cytosolic mouth of the channel. It has been known for many years that $InsP_3Rs$ are under a biphasic regulation by the local $[Ca^{2+}]_c$, with submicromolar $[Ca^{2+}]$ being required for activation and supramicromolar $[Ca^{2+}]$ causing inhibition (Bezprozvanny *et al.* 1991; Kaftan *et al.* 1997; Miyakawa *et al.* 2001). This positive and negative feedback regulation appears to be a key element responsible of the production of regenerative Ca^{2+} oscillations (Putney & Bird, 1993; Fewtrell, 1993; Berridge & Dupont, 1994; Miyakawa *et al.* 2001; Hattori *et al.* 2004; Patterson *et al.* 2004) and mitochondria have been shown before to modulate $InsP_3$-induced Ca^{2+} release by acting on this mechanism. In hepatocytes, block of mitochondrial Ca^{2+} uptake increased Ca^{2+} release, suggesting that mitochondria were suppressing the local feedback activation by Ca^{2+} of $InsP_3Rs$ (Hajnoczky *et al.* 1999). In HeLa cells, instead, block of mitochondrial Ca^{2+} uptake with uncouplers inhibited histamine-induced Ca^{2+} release and oscillations (Collins *et al.* 2000), perhaps because of the increased feedback inhibition by Ca^{2+} of $InsP_3Rs$ in the absence of Ca^{2+} uptake by nearby mitochondria. In fact, feedback inhibition by Ca^{2+} in these cells is the main mechanism limiting histamine-induced Ca^{2+} release, as histamine induces a fast and complete Ca^{2+} release from the ER in cells loaded with BAPTA (Montero *et al.* 1997). The effect of MCU activation increasing ER Ca^{2+} release in HeLa cells (Fig. 1) is therefore best explained as a result of the reduced feedback inhibition by Ca^{2+} of $InsP_3R$ following the increase in mitochondrial Ca^{2+} uptake. It is interesting to note that MCU activation produced little increase in the mean $[Ca^{2+}]_m$, except when MNCE was simultaneously inhibited (Fig. 5). This suggests that MNCE rapidly extrudes the increased Ca^{2+} intake in the presence of the activators, so that the mean $[Ca^{2+}]_m$ is little changed. However, if MNCE is inhibited, the increased mitochondrial Ca^{2+} uptake that is induced by PPT during Ca^{2+} oscillations, accumulates and results in a much larger mean $[Ca^{2+}]_m$.

Our data therefore suggest that MCU activation potentiates histamine-induced Ca^{2+} release from the ER by reducing feedback inhibition of $InsP_3Rs$ by Ca^{2+}. This is consistent with the reported inhibition of ER Ca^{2+} release after block of mitochondrial Ca^{2+} uptake with uncouplers in HeLa cells (Collins *et al.* 2000). Evidence for the direct interaction between mitochondria and ER has been obtained before from the observation of close physical contacts between both organelles and from the observation that mitochondria take up Ca^{2+} much more effectively after $InsP_3$-induced Ca^{2+} release than after global homogeneous increases in $[Ca^{2+}]$ (Rizzuto *et al.* 1998; Csordas *et al.* 1999). In addition, there is also evidence that these close couplings between mitochondria and ER facilitate Ca^{2+} transfer from mitochondria to the ER via MNCE releasing Ca^{2+} close to ER Ca^{2+} pumps (Arnaudeau *et al.* 2001; Malli *et al.* 2003, 2005). In a similar way, we have recently shown that inhibition of MNCE potentiates Ca^{2+} release from the ER (Hernández-SanMiguel *et al.* 2006). This suggested that MNCEs are placed close to $InsP_3Rs$, so that Ca^{2+} release from mitochondria through this system would be able to generate or maintain the local $[Ca^{2+}]_c$ microdomain around $InsP_3Rs$ necessary to produce feedback inhibition. Our data here suggest that MCUs are also able to modulate that local $[Ca^{2+}]_c$ microdomain and should therefore also be close to $InsP_3Rs$. In fact, we have shown that both MCU activation and

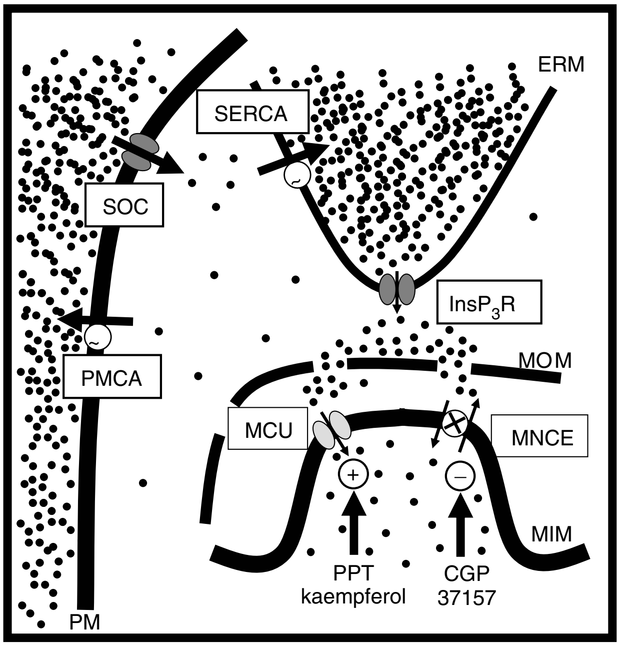

Figure 9. A putative model of the local interactions between mitochondrial Ca^{2+} uniporter (MCU), mitochondrial Na^+–Ca^{2+} exchanger (MNCE) and endoplasmic reticulum inositol 1,4,5-trisphosphate receptor ($InsP_3R$)

Ca^{2+} released through $InsP_3R$ is rapidly transported into mitochondria via MCU and then extruded from this organelle through MNCE. 4,4′,4″-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT) activates MCU and CGP37157 inhibits MNCE, so in the presence of both drugs, Ca^{2+} is accumulated into mitochondria thus reducing the size of the local cytosolic $[Ca^{2+}]$ microdomain around $InsP_3R$. The plasma membrane (PMCA) and endoplasmic reticulum (ER) Ca^{2+} pumps (SERCA) restore cytosolic and ER $[Ca^{2+}]$ to resting levels in the intervals between oscillations. For this, Ca^{2+} entry through store-operated channels (SOCs) is also essential. ERM, ER membrane; MOM, mitochondrial outer membrane; MIM, mitochondrial inner membrane; PM plasma membrane.

MNCE inhibition produce additive effects in terms of activating Ca^{2+} release (Fig. 1*B*). It is interesting to note, however, that MNCE inhibition and MCU activation do not produce additive effects on Ca^{2+} oscillations. Instead, MNCE inhibition enhances oscillations and subsequent MCU activation inhibits them, usually after an initial burst. The most probable explanation for this apparent paradox is the excessive Ca^{2+} depletion induced by the over-stimulation of Ca^{2+} release (see Fig. 1), which may preclude further spiking. Both MNCE inhibition and MCU activation would cooperate to reduce the local Ca^{2+} accumulation around $InsP_3Rs$, thus avoiding feedback Ca^{2+} inhibition of Ca^{2+} release and leading to prolonged stimulation of Ca^{2+} release. In conclusion, our data suggest that $InsP_3Rs$ from the ER and MNCEs and MCUs from mitochondria colocalize in the small subcellular regions where ER and mitochondria form close contacts. In these functional units both MCUs and MNCEs finely tune the local $[Ca^{2+}]_c$ microdomain to modulate ER Ca^{2+} release. Figure 9 shows a schematic model of these interactions, which also includes the recycling of Ca^{2+} through the plasma membrane which is required to maintain oscillations. When cells are activated, $InsP_3$ activates Ca^{2+} release from the ER until feedback Ca^{2+} inhibition of $InsP_3R$ develops. Then, the $[Ca^{2+}]_c$ transient is terminated by the action of plasma membrane and ER Ca^{2+} pumps and the ER is refilled with Ca^{2+} entering the cell through plasma membrane store-operated Ca^{2+} channels. Once the ER is again full of Ca^{2+} and $[Ca^{2+}]_c$ has returned close to resting levels, a new oscillation may appear if $InsP_3$ is still present. As we have shown in this paper, and previously (Hernández-SanMiguel *et al.* 2006), the balance between the rates of Ca^{2+} uptake and release from mitochondria modulates feedback Ca^{2+} inhibition and thus oscillations. In addition, other parameters of the model may also modulate oscillations. It has been shown before that changes in extracellular $[Ca^{2+}]$, and thus changes in Ca^{2+} entry rate, also affect the frequency of the oscillations (Bootman *et al.* 1996). We should also mention here that ryanodine receptors, although scarcely present in HeLa cells (Bennett *et al.* 1996), are also sensitive to local cytosolic Ca^{2+} levels and their interaction with mitochondria may play a role in the modulation of Ca^{2+} oscillations in these and other cells. Therefore, the Ca^{2+} spike frequency appears to be finely modulated by most of the Ca^{2+} fluxes shown in the model of Fig. 9.

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Acknowledgements

This work was supported by grants from Ministerio de Educación y Ciencia (BFU2005-05464), from Junta de Castilla y León (VA016A05) and from Fondo de Investigaciones Sanitarias (PI040789). J.S. and E.H.-S. hold research personnel training (FPI) and University personnel training (FPU) fellowships, respectively, from the Spanish Ministerio de Educación y Ciencia. L.V. holds a fellowship from Fondo de Investigaciones Sanitarias (Spanish Ministerio de Sanidad). We thank Elena González for excellent technical assistance.