ELSEVIER

Cell Calcium 40 (2006) 53–61

cell calcium

www.elsevier.com/locate/ceca

The mitochondrial Na^+/Ca^{2+} exchanger plays a key role in the control of cytosolic Ca^{2+} oscillations

Esther Hernández-SanMiguel, Laura Vay, Jaime Santo-Domingo, Carmen D. Lobatón, Alfredo Moreno, Mayte Montero, Javier Alvarez *

Instituto de Biología y Genética Molecular (IBGM), Departamento de Bioquímica y Biología Molecular y Fisiología, Facultad de Medicina, Universidad de Valladolid and Consejo Superior de Investigaciones Científicas (CSIC), Ramón y Cajal, 7, E-47005 Valladolid, Spain

Received 23 January 2006; received in revised form 6 March 2006; accepted 23 March 2006

Abstract

There is increasing evidence that mitochondria play an important role in the control of cytosolic Ca^{2+} signaling. We show here that the main mitochondrial Ca^{2+}-exit pathway, the mitochondrial Na^+/Ca^{2+} exchanger, controls the pattern of cytosolic Ca^{2+} oscillations in non-excitable cells. In HeLa cells, the inhibitor of the mitochondrial Na^+/Ca^{2+} exchanger CGP37157 changed the pattern of the oscillations induced by histamine from a high-frequency irregular one to a lower frequency baseline spike type, surprisingly with little changes in the average Ca^{2+} values of a large cell population. In human fibroblasts, CGP37157 increased the frequency of the baseline oscillations in cells having spontaneous activity and induced the generation of oscillations in cells without spontaneous activity. This effect was dose-dependent, disappeared when the inhibitor was washed out and was not mimicked by mitochondrial depolarization. CGP37157 increased mitochondrial $[Ca^{2+}]$ and ATP production in histamine-stimulated HeLa cells, but the effect on ATP production was only transient. CGP37157 also activated histamine-induced Ca^{2+} release from the endoplasmic reticulum and increased the size of the cytosolic Ca^{2+} peak induced by histamine in HeLa cells. Our results suggest that the mitochondrial Na^+/Ca^{2+} exchanger directly modulates inositol 1,4,5-trisphosphate-induced Ca^{2+} release and in that way controls cytosolic Ca^{2+} oscillations.
© 2006 Elsevier Ltd. All rights reserved.

Keywords: Calcium; CGP37157; Aequorin; Na^+/Ca^{2+} exchange; Mitochondria; HeLa; Fibroblasts

1. Introduction

There is much evidence that mitochondria modulate cytosolic Ca^{2+} signaling and has a privileged relationship in terms of Ca^{2+} homeostasis with both the endoplasmic reticulum and the plasma membrane. The large capacity and rate of Ca^{2+} accumulation by mitochondria allows this organelle to accumulate transiently most of the Ca^{2+} influx occurring during cell stimulation [1]. Thus, acting as transient Ca^{2+} buffers, mitochondria are able to modulate the size of the cytosolic Ca^{2+} ($[Ca^{2+}]_c$) transient and the associated physiological phenomena, such as secretion [2,3]. The subsequent slower release of the accumulated Ca^{2+} by mitochondria generates characteristic shoulders in the stimulus-induced $[Ca^{2+}]_c$ transients [4–6] and facilitates also refilling with Ca^{2+} of the endoplasmic reticulum [7–9] when the Ca^{2+} transient is due to Ca^{2+}-release from this organelle. There is also structural and functional evidence suggesting that mitochondria and ER are closely coupled in terms of Ca^{2+} homeostasis, probably through specific and stable interactions among both organelles [10–13]. In that way, the close proximity between inositol 1,4,5-trisphosphate receptors ($InsP_3R$) and mitochondrial Ca^{2+} uniporters may facilitate a rapid transfer of Ca^{2+} from the ER to the mitochondria, as well as the modulation by mitochondria of the local Ca^{2+} microenvironment surrounding $InsP_3R$. This allows mitochondria

Abbreviations: $[Ca^{2+}]_c$, cytosolic $[Ca^{2+}]$; $[Ca^{2+}]_M$, mitochondrial $[Ca^{2+}]$; ER, endoplasmic reticulum; $[Ca^{2+}]_{ER}$, ER $[Ca^{2+}]$; $InsP_3$, inositol 1,4,5-trisphosphate; $InsP_3R$, $InsP_3$ receptor

* Corresponding author. Tel.: +34 983 423085; fax: +34 983 423588.
E-mail address: jalvarez@ibgm.uva.es (J. Alvarez).

0143-4160/$ – see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ceca.2006.03.009

to control Ca^{2+} release, as $InsP_3R$ have a strong positive and negative feedback regulation by the local $[Ca^{2+}]$ surrounding the release site [14,15]. In addition, mitochondria appear to hold also specific interactions with the plasma membrane, where they regulate the opening of different Ca^{2+} channels by maintaining a low-Ca^{2+} environment close to the channels, thus avoiding their inactivation by Ca^{2+} [16,17].

The role of mitochondria in cytosolic Ca^{2+} signaling has been widely explored using protonophores or respiratory chain inhibitors to depolarize the mitochondrial membrane and thus block Ca^{2+} uptake into the organelle. Mitochondrial depolarization usually induces a large increase in the size of the stimulus-induced $[Ca^{2+}]_c$ transient and accelerates the return of $[Ca^{2+}]_c$ to resting levels [4,6,18,19]. In addition, this maneuver has been shown to inhibit the generation of long-lasting Ca^{2+} oscillations [20] and to produce a larger ER depletion [7,8] in stimulated HeLa cells. On the other hand, Ca^{2+} efflux from mitochondria occurs mainly through the mitochondrial Na^+/Ca^{2+} exchanger and the specific inhibitor of this transport system CGP37157 has also been used to investigate the relationship between mitochondrial and cytosolic Ca^{2+} homeostasis. This inhibitor has been shown to mimic the effect of mitochondrial depolarization by reducing ER Ca^{2+}-refilling in non-excitable cells stimulated with an $InsP_3$-producing agonist [7,9]. Thus, the mitochondrial Na^+/Ca^{2+} exchanger has been implicated in both ER-mitochondria Ca^{2+} recycling [7] and in the transfer of Ca^{2+} from the extracellular medium to the ER through mitochondria [8,9].

We have studied here further the role of the mitochondrial Na^+/Ca^{2+} exchanger in cytosolic and mitochondrial Ca^{2+} homeostasis and we show evidence that this system has a distinct role in the modulation of $InsP_3$-induced cytosolic Ca^{2+} oscillations, thus suggesting a functional coupling between the exchanger and $InsP_3R$.

2. Experimental procedures

2.1. Cell culture and targeted aequorin expression

HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum. The constructs for aequorin targeted to the cytosol and mutated aequorin targeted to either the ER or the mitochondria have been described previously [3,21]. The luciferase gene was obtained from Promega (pSP-luc + vector) and cloned into the pcDNA3.1 plasmid. The HeLa cell clone MM5, that stably expresses mitochondrially-targeted mutated aequorin has also been described previously [22]. Transfections were carried out using Metafectene (Biontex, Munich, Germany). Cultures of human fibroblasts were obtained from skin biopsies of healthy donor volunteers. They were grown in 199 medium supplemented with 10% fetal calf serum.

2.2. $[Ca^{2+}]_M$, $[Ca^{2+}]_c$, and $[Ca^{2+}]_{ER}$ measurements in cell populations with targeted aequorin

In the case of the mitochondrial $[Ca^{2+}]$ measurements, a mutated form of aequorin with lower Ca^{2+} affinity was used in order to reduce aequorin consumption and thus measure better the real changes in $[Ca^{2+}]_M$, avoiding the artifacts due to saturation with Ca^{2+} of aequorin. A detailed discussion of the reasons for using a low-affinity aequorin to measure $[Ca^{2+}]_M$ changes has been presented elsewhere [22]. The HeLa cell clone MM5 was used for most of the measurements of mitochondrial $[Ca^{2+}]$ ($[Ca^{2+}]_M$). In some cases, $[Ca^{2+}]_M$ measurements were made using wild type HeLa cells transfected with the pcDNA3.1 plasmid containing the construct for mitochondrially-targeted mutated aequorin. Cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_c$) measurements were carried out using HeLa cells transiently transfected with the plasmid for cytosolic aequorin. Cells were plated onto 13 mm round coverslips. For aequorin reconstitution, HeLa cells expressing cytosolic aequorin were incubated for 1–2 h at room temperature with 1 μM of wild-type coelenterazine, and cells expressing mitochondrially-targeted mutated aequorin were incubated for 1–2 h at room temperature with either 1 μM of wild-type coelenterazine (for experiments with intact cells) or 1 μM of coelenterazine n (for experiments with permeabilized cells), in standard medium containing 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES, pH 7.4. Cells were then placed in the perfusion chamber of a purpose-built luminometer thermostatized at 37 °C. For the experiments with permeabilized cells, mitochondrially-targeted mutated aequorin was reconstituted with coelenterazine n in order to reduce still further its Ca^{2+}-affinity, thus allowing measurement of higher $[Ca^{2+}]_M$. Then, standard medium containing 0.5 mM EGTA instead of Ca^{2+} was perfused for 1 min, followed by 1 min of intracellular medium (130 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, 1 mM K_3PO_4, 0.5 mM EGTA, 1 mM ATP, 20 μM ADP, 2 mM succinate, 20 mM Hepes, pH 7) containing 100 μM digitonin and again intracellular medium without digitonin for 5–10 min prior to the addition of $[Ca^{2+}]$ buffers. 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 and endoplasmic reticulum Ca^{2+}-ATPase inhibitor 2,5-di-*tert*-buthyl-benzohydroquinone (BHQ) 10 μM in standard external medium containing (in mM): NaCl, 145; KCl, 5; $MgCl_2$, 1; glucose, 10; 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. Then, the coverslip was placed in the perfusion chamber of a purpose-built thermostatized luminometer, and standard medium containing 0.5 mM EGTA was perfused for 5 min prior to the experiment.

2.3. Measurements of intracellular ATP

HeLa cells expressing the plasmid for cytosolic luciferase were placed in the perfusion chamber of a purpose-built luminometer thermostatized at 37 °C. Then, standard medium containing 2–5 μM luciferin was perfused until a steady-state luminescence was reached. Then, the required stimuli were perfused in standard medium containing the same amount of luciferin.

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

HeLa cells or fibroblasts were loaded with fura-2 by incubation in standard medium containing 2 μM 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 in the stage of a Zeiss Axiovert 200 microscope under continuous perfusion. Single cell fluorescence was excited at 340 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 by a Hamamatsu ORCA-ER camera. Single cell fluorescence records were ratioed and calibrated into $[Ca^{2+}]$ values off-line as described before [23] using the Metafluor program (Universal Imaging). Experiments were performed at 37 °C using an on-line heater from Harvard Apparatus.

2.5. Materials

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

3. Results

3.1. Dose-dependence of the inhibition by CGP37157 of mitochondrial Ca^{2+} exit

The mitochondrial Na^+/Ca^{2+} exchanger is the main system responsible of mitochondrial Ca^{2+}-efflux in most cell types. Fig. 1 shows that Ca^{2+}-efflux from mitochondria

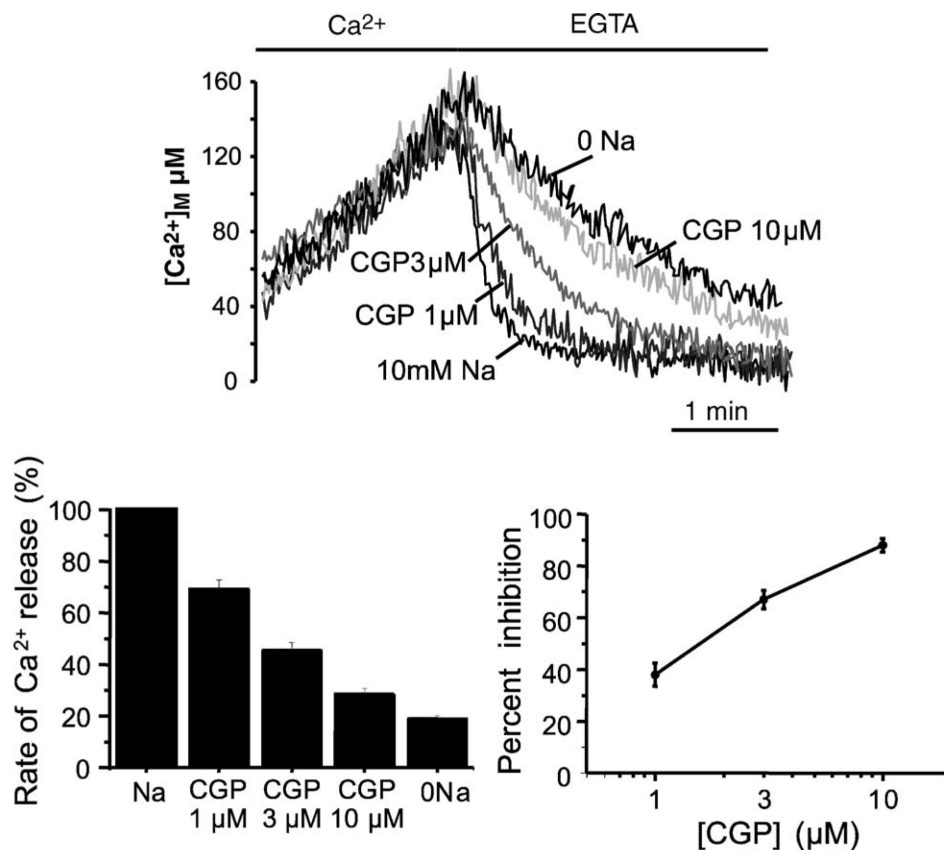

Fig. 1. Inhibition by CGP37157 of mitochondrial Ca^{2+} release. MM5 cells expressing mutated mitochondrially-targeted aequorin were reconstituted with coelenterazine n. Then, they were permeabilized as described in Section 2 and a Ca^{2+} buffer containing 2.5 μM $[Ca^{2+}]$ in Na^+-free medium was perfused as indicated in the figure. Then, Ca^{2+} release from mitochondria was triggered by perfusing medium containing 0.5 mM EGTA instead of the $[Ca^{2+}]$ buffer. In addition, 10 mM Na^+ was included at that point in all the experiments (except that labeled "0 Na") either in the absence or in the presence of 1, 3 or 10 μM CGP37157, as indicated (CGP37157 was present also during the Ca^{2+} uptake period in those experiments). The lower panel show mean data obtained from 8 to 17 experiments of each kind similar to those shown in the upper panel.

in permeabilized HeLa cells is largely inhibited when medium containing no Na^+ is perfused. Cells expressing mitochondrially-targeted aequorin were permeabilized as described in Section 2 and then mitochondrial Ca^{2+} uptake was induced by perfusing a 2.5 μM Ca^{2+}-buffer in Na^+-free intracellular medium (140 mM KCl, 1 mM $MgCl_2$, 1 mM K_3PO_4, 0.5 mM potassium EGTA, 1 mM potassium ATP, 2 mM potassium succinate, 20 mM Hepes, pH 7 with KOH). Then, release of the accumulated Ca^{2+} was triggered by perfusing medium without Ca^{2+} (EGTA-containing) and with either 0 or 10 mM Na^+. In several similar experiments, the rate of Ca^{2+} release obtained in the presence of Na^+-free medium was 19 ± 1% (mean ± S.E., $n = 15$) of that obtained in Na^+-containing medium. The figure shows also that CGP37157, an inhibitor of the mitochondrial Na^+/Ca^{2+} exchanger, produced a dose-dependent inhibition of the rate of Ca^{2+} release in Na^+-containing medium. The lower panels show the dose-dependence of this inhibition. Nearly-full inhibition was obtained at 10 μM (88 ± 3% of the effect of Na^+-free medium, mean ± S.E., $n = 15$), and half-maximal inhibition at 1.6 ± 1.0 μM (mean ± S.D., sigmoidal logEC50 fitting, BioDataFit program, Chang Bioscience, CA, USA). The residual Ca^{2+} release from mitochondria observed in the absence of Na^+ may take place through Na^+-independent Ca^{2+} extrusion pathways, such as the H^+/Ca^{2+} exchanger [24].

3.2. *Effects of CGP37157 on $[Ca^{2+}]_c$, $[Ca^{2+}]_M$ and $[Ca^{2+}]_{ER}$ responses to histamine*

Our main objective in this paper was to investigate if modulation of the activity of the mitochondrial Na^+/Ca^{2+} exchanger significantly affects cytosolic Ca^{2+} homeostasis. During cytosolic Ca^{2+}-transients induced by agonists, part of the Ca^{2+} released from the endoplasmic reticulum is transiently accumulated into mitochondria. Inhibition of the mitochondrial Na^+/Ca^{2+} exchanger should increase the amount of Ca^{2+} accumulated into mitochondria and thus reduce the $[Ca^{2+}]$ in the cytosol. We first studied to what extent was CGP37157 able to modify the mitochondrial Ca^{2+} peaks induced by an agonist. Fig. 2, upper panel, shows that CGP37157 increased both the size and the duration of the $[Ca^{2+}]_M$ peak induced by histamine. However, as expected for an inhibitor of mitochondrial Ca^{2+} efflux, the effect on the duration of the $[Ca^{2+}]_M$ transient was much larger than that on the peak values. The $[Ca^{2+}]_M$ peak was increased from 22 ± 2 μM in the control to 32 ± 2 μM (mean ± S.E., $n = 15$ in both cases) in the presence of CGP37157 10 μM, an increase of near 50%. The duration of the peak was estimated indirectly by looking at the $[Ca^{2+}]_M$ level 2 min after start of the stimulation. Under control conditions, $[Ca^{2+}]_M$ rapidly returned to resting levels after the peak, and 2 min after stimulation it was only 0.77 ± 0.11 μM (resting levels before stimulation 0.23 ± 0.03 μM). Instead, in the presence of CGP37157 10 μM, $[Ca^{2+}]_M$ required a much longer time to return to resting levels and the $[Ca^{2+}]_M$ level 2 min after start of the stimulation was 3.76 ± 0.31 μM, about five-fold higher (mean ± S.E., $n = 15$ in all cases). We expected that these effects of CGP37157 on the $[Ca^{2+}]_M$ peak induced by histamine would be accompanied by a corresponding decrease in the $[Ca^{2+}]_c$ peak. However, the cytosolic $[Ca^{2+}]$ peak was slightly but significantly increased in the presence of CGP37157, from 1.02 ± 0.04 μM in the controls to 1.15 ± 0.03 μM in the presence of the inhibitor (mean ± S.E., $n = 11$ in both cases, $p < 0.05$, ANOVA test). Fig. 2, lower panel, shows mean data of 11 separate experiments of each kind, and the differences can be better seen in the right trace when both peaks are superimposed. The figure shows that, in addition to the increase in the $[Ca^{2+}]_c$ peak, the rate of $[Ca^{2+}]_c$ decrease after the peak was also increased in the presence of CGP37157, so that $[Ca^{2+}]_c$ in the shoulder of the transient was significantly smaller ($p < 0.05$ for the differences in the mean values along the shoulder) in the presence of this compound. This effect is consistent with the increased accumulation of Ca^{2+} in mitochondria.

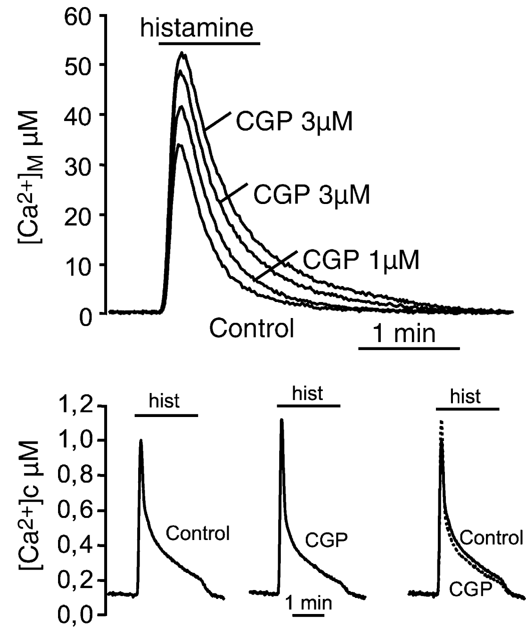

Fig. 2. Effect of CGP37157 on the histamine-induced mitochondrial and cytosolic $[Ca^{2+}]$ peaks. Upper panel: MM5 cells expressing mutated mitochondrially-targeted aequorin were reconstituted with native coelenterazine and stimulated with 100 μM histamine either in the presence or in the absence of several concentrations of CGP37157, as indicated. This compound was added in each case 2 min prior to the addition of histamine. Experiments are representative of 7–15 similar experiments of each kind. Lower panel: HeLa cells transfected with cytosolic aequorin were reconstituted with native coelenterazine. Then, they were stimulated with 100 μM histamine either in the absence or in the presence of 10 μM CGP37157 (preincubated for 2 min prior to histamine stimulation). The traces shown are the mean of 11 experiments of each type. The right trace shows the two experiments superimposed (dotted trace with CGP37157).

The discrepancy among the changes induced by CGP37157 in the mitochondrial and cytosolic $[Ca^{2+}]$ peaks could be due to effects of the inhibition of mitochondrial Na^+/Ca^{2+} exchanger by CGP37157 on the release of Ca^{2+} from the endoplasmic reticulum. It has been previously

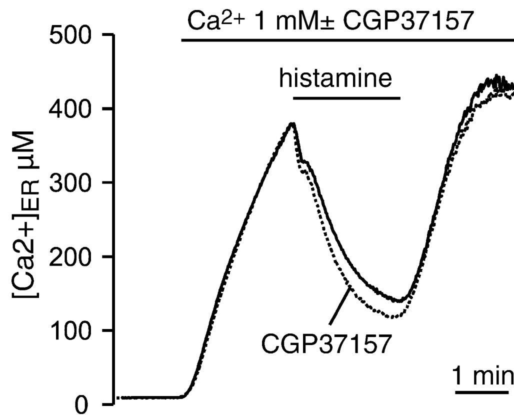

Fig. 3. Effect of CGP37157 on histamine-induced Ca^{2+} release from the ER. HeLa cells transfected with ER-targeted aequorin were depleted of Ca^{2+} and reconstituted with coelenterazine n as described in Section 2. Then, medium containing 1 mM Ca^{2+} in the presence or in the absence of 10 μM CGP37157 was perfused to refill the ER with Ca^{2+}. When indicated, cells were stimulated with 100 μM histamine. Experiments shown are the mean of five different experiments and are representative of 11 similar ones of each kind.

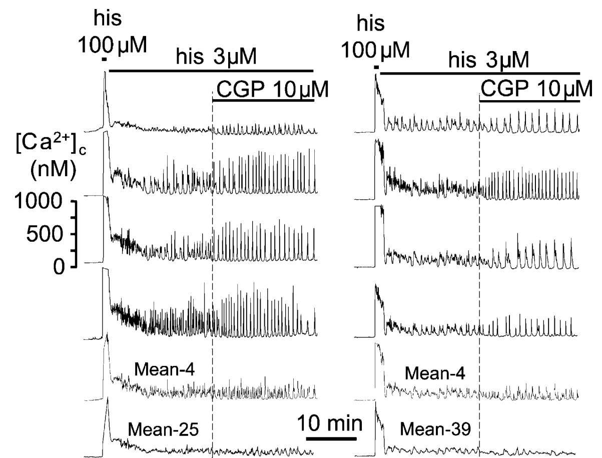

Fig. 4. Effect of CGP37157 on histamine-induced $[Ca^{2+}]_c$ oscillations in HeLa cells. Fura-2-loaded HeLa cells were stimulated for 1 min with 100 μM histamine and then for the rest of the experiment with 3 μM histamine. When indicated, medium containing also 10 μM CGP37157 was perfused. The figure shows data from two different experiments: four single-cell records, the mean of these four records (mean-4) and the mean of all the cells present in the microscope field in each case (mean-25 or mean-39). These experiments are representative of 20 similar ones.

shown that CGP37157 increases ER-Ca^{2+} depletion in histamine-stimulated HeLa cells [7], but that effect was only attributed to a decreased ER refilling. We have used aequorin targeted to the endoplasmic reticulum to investigate in detail the kinetics of the effect of CGP37157 on the Ca^{2+} release induced by histamine. Fig. 3 shows that, as reported previously [25], Ca^{2+} release from the ER is biphasic, with a fast initial step of Ca^{2+} release that suddenly stops and is followed by a second phase of slower release. As reported previously [7], in the presence of CGP37157 release of Ca^{2+} was significantly increased. Considering the amount of Ca^{2+} released in the initial 90s after stimulation with histamine, the ER released 60.5 ± 2.0% (mean ± S.E., n = 11) of its content in the controls and 68.9 ± 1.5% (mean ± S.E., n = 11) of its content in the presence of CGP37157 10 μM ($p < 0.005$, ANOVA test). However, the increase in the rate of release started already in the initial fast phase of release. Measuring the amount of Ca^{2+} released during the initial 10 s after stimulation, the ER released 17.8 ± 1.0% (mean ± S.E., n = 11) of its content in the controls and 21.6 ± 1.2% (mean ± S.E., n = 11) of its content in the presence of CGP37157 10 μM ($p < 0.05$, ANOVA test). These results suggest that CGP37157 may also directly stimulate Ca^{2+} release, at least during the initial fast phase of release. This larger Ca^{2+} release from the ER may compensate for the increased accumulation of Ca^{2+} in mitochondria, thus explaining the observed increase in the cytosolic $[Ca^{2+}]$ peak induced by histamine in the presence of CGP37157.

3.3. Effects of CGP37157 on $[Ca^{2+}]_c$ oscillations in HeLa cells and human fibroblasts

The increased Ca^{2+}-release from the ER in the presence of CGP37157 was surprising, because it suggested that Ca^{2+} release from mitochondria through the Na^+/Ca^{2+} exchanger could modulate the activity of $InsP_3R$. If that was the case, we reasoned that mitochondrial Na^+/Ca^{2+} exchanger could also modulate cytosolic Ca^{2+} oscillations. To test this hypothesis, we have used two different cell models: histamine-stimulated HeLa cells and spontaneous oscillations in primary fibroblasts. In both cases $[Ca^{2+}]_c$ oscillations derive from periodic Ca^{2+} release from the endoplasmic reticulum via $InsP_3R$, as these cells do not possess voltage-dependent Ca^{2+} channels in the plasma membrane. This is important to validate the effects obtained with CGP37157, as this compound has been reported to inhibit also voltage-dependent Ca^{2+} channels [26].

Fig. 4 shows single HeLa cell records of cytosolic Ca^{2+} from two different experiments, together with the mean response of the four cells shown (mean-4) and that of all the cells present in the microscope field (lower trace in each case). A low concentration of histamine (3 μM) was used to facilitate the generation of long-lasting oscillations. We have shown previously that this concentration produces a much slower release of Ca^{2+} from the ER [25]. HeLa cells were stimulated with histamine to trigger Ca^{2+} oscillations and then the mitochondrial Na^+/Ca^{2+} exchange was inhibited by perfusing CGP37157. It can be observed that inhibition of mitochondrial Na^+/Ca^{2+} exchange produces a clear change in the oscillatory pattern in the single cells, although surprisingly the mean values were not significantly modified. The mean of the four single-cell traces shows already that, in spite of the dramatic change in the oscillatory pattern, mean $[Ca^{2+}]$ values are scarcely affected even when only a few cells are averaged. Consistently, in the mean of all the cells present in the microscope field (lower trace in each experiment), CGP37157 produces no effect at all. Careful examination of

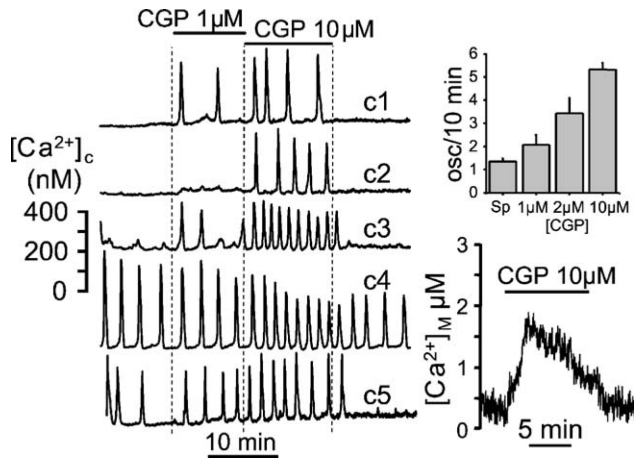

Fig. 5. Effect of CGP37157 on $[Ca^{2+}]_c$ oscillations in human fibroblasts. Fura-2 loaded human fibroblasts were perfused with standard medium containing either no additions, 1 or 10 μM CGP37157, as indicated. The left panel shows five single-cell records belonging to five cells present in the same microscope field. These experiments are representative of 30 similar ones. The upper right panel shows statistical data of the increase in the frequency of oscillations induced by several concentrations of CGP37157 (Sp = spontaneous oscillations). The number of cells analyzed for each condition was: spontaneous, 157; CGP37157 1 μM, 13; CGP37157 2 μM, 14; CGP37157 10 μM, 54. In the lower right panel, fibroblasts expressing mitochondrially-targeted mutated aequorin were reconstituted with coelenterazine and stimulated with 10 μM CGP37157. This experiment is representative of 10 similar ones.

the single cell traces provides an explanation for this apparent contradiction. In the absence of CGP37157, oscillations are faster and most times Ca^{2+} does not return to resting levels in the interval between two consecutive spikes. In contrast, in the presence of CGP37157, the size of the oscillations increases, the frequency is reduced and $[Ca^{2+}]_c$ fully returns to resting levels before the start of a new spike. The increase in size of the spikes compensates the decrease in frequency and in inter-spike $[Ca^{2+}]$ level, so that the average of many different cells with non-synchronic oscillations is not modified.

In Fig. 5, we can see single cell $[Ca^{2+}]_c$ measurements in human fibroblasts. In some cells, spontaneous $[Ca^{2+}]_c$ oscillations were already observed in the absence of stimulation (see cells 4 and 5). These spontaneous oscillations were observed in 52% of the cells (of 157 analyzed cells), while the rest were silent under resting conditions, at least for the initial 10 min of recording. In any case, addition of CGP37157 induced the generation of $[Ca^{2+}]_c$ oscillations in those cells previously silent or increased the frequency of the oscillations in those cells showing spontaneous oscillations. The experiments shown reveal that this effect was induced by submaximal concentrations of CGP37157 (1 μM), and the oscillatory frequency obtained increased when the CGP37157 concentration was increased. The concentration-dependence of this effect of CGP37157 is shown in the upper right panel of the figure and was very similar to that obtained above (Fig. 1) for the inhibition of Ca^{2+} release from mitochondria by this compound, suggesting that both effects share a common mechanism. The frequency of the $[Ca^{2+}]$ oscillations measured as oscillations/10 min increased from 1.35 ± 0.13 in the absence of CGP37157 to 2.08 ± 0.41, 3.43 ± 0.66 and 5.32 ± 0.29 in the presence of 1, 2 and 10 μM CGP37157, respectively (mean ± S.E., the number of data is shown in the figure). This increase in the frequency of $[Ca^{2+}]_c$ oscillations occurred in parallel with an increase in $[Ca^{2+}]_M$ in the low micromolar range (lower right panel of Fig. 5).

3.4. Effect of CGP37157 on $[Ca^{2+}]_M$ and ATP in histamine-stimulated HeLa cells

Finally, to obtain further information on the mechanism of activation of Ca^{2+} oscillations by CGP37157, we have tested if this compound modified the intracellular ATP levels. Fig. 6, upper panel, shows that CGP37157 produces a prolonged increase in mitochondrial $[Ca^{2+}]$ in HeLa cells stimulated with 5 μM histamine. The presence of histamine is necessary to trigger Ca^{2+} oscillations in HeLa cells, so that mitochondria may take up Ca^{2+}. In the absence of histamine, CGP37157 produces no increase in mitochondrial Ca^{2+} in these cells (data not shown). It is known that an increase in mitochondrial $[Ca^{2+}]$ activates ATP production [27], and ATP is known to modulate the inositol 1,4,5-trisphosphate

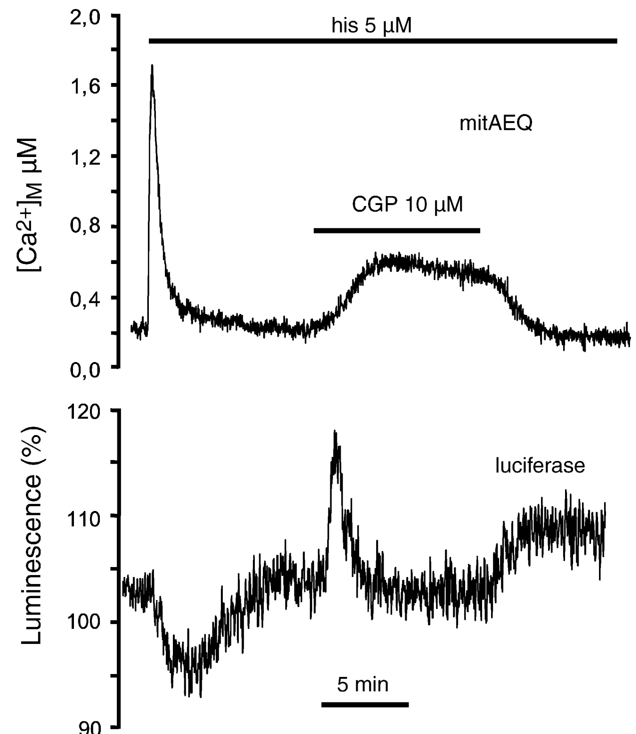

Fig. 6. Effects of CGP37157 on $[Ca^{2+}]_M$ and cytosolic ATP in histamine-stimulated HeLa cells. Upper panel: MM5 cells expressing mutated mitochondrially-targeted aequorin were reconstituted with native coelenterazine and stimulated with 5 μM histamine. When indicated, 10 μM CGP37157 was perfused still in the presence of histamine. Lower panel: HeLa cells expressing cytosolic luciferase were perfused with 2 μM luciferin and the same stimuli as in the upper panel. These experiments are representative of 15 similar ones of each kind.

receptor [28]. Therefore, we wanted to test if the ATP levels were increased along this period. Fig. 6, lower panel, shows a record of luminescence obtained in cells expressing luciferase in the cytosol and perfused with luciferin to measure intracellular levels of ATP on-line. As described previously [27], stimulation with histamine in glucose-containing medium induces a decrease in ATP levels due to the activation of Ca^{2+}-pumps in the plasma membrane and in the endoplasmic reticulum. This excess ATP waste cannot be compensated by the increased ATP production due to the activation of respiration by the increased mitochondrial Ca^{2+}. Then, the steady-state ATP levels return to the initial state while still in the presence of histamine, once the increased ATP production compensates for the waste due to Ca^{2+}-pumping during the oscillations. At this point, addition of CGP37157 produces a fast but surprisingly very transient increase in ATP concentration. The ATP levels then return rapidly to the values present before CGP37157 addition and keep constant all the time while CGP37157 is present. Interestingly, when CGP37157 is washed at the end of the experiment, ATP levels increase again.

4. Discussion

We show in this paper that inhibition of the mitochondrial Na^+/Ca^{2+} exchanger with the specific inhibitor CGP37157 produces dramatic changes in the pattern of cytosolic Ca^{2+} oscillations in non-excitable cells. In HeLa cells, inhibition of the mitochondrial Na^+/Ca^{2+} exchanger shifted the pattern from high-frequency irregular oscillations to baseline spikes and in fibroblasts increased the frequency of the oscillations or induced the generation of spikes in cells previously silent. These effects are not mimicked by a global abolition of the mitochondrial Ca^{2+} buffering capacity. It has been shown that mitochondrial depolarization inhibits the production of Ca^{2+} oscillations in HeLa cells [20] and we have seen here that the same occurs in fibroblasts (data not shown). Therefore, our data suggest a specific role for the mitochondrial Na^+/Ca^{2+} exchange in the regulation of $InsP_3$-dependent $[Ca^{2+}]_c$ signaling.

It has been known for many years that binding of $InsP_3$ to the $InsP_3R$ is necessary but not sufficient to open the channel, and Ca^{2+} binding to a specific region of the $InsP_3R$ [29] is required as a coagonist for the opening of the channel [14,15]. In addition, the effect of Ca^{2+} on the $InsP_3R$ is biphasic, producing inhibition of Ca^{2+} release at higher concentrations. Therefore, $InsP_3$-induced Ca^{2+} release is under both positive and negative feedback control by the released Ca^{2+} and this modulation appears to be critical for the development of Ca^{2+} signaling patterns such as oscillations or waves [29–33]. It has been shown previously that mitochondria closely associated to the ER inhibit Ca^{2+} release through $InsP_3R$ [10] and the suggested mechanism involves Ca^{2+} uptake by mitochondria suppressing the local positive feedback effects of Ca^{2+} on the $InsP_3R$. Consistently, the presence of close couplings between mitochondrial Ca^{2+} uptake sites and $InsP_3R$ has been inferred from the fact that mitochondria take up Ca^{2+} much more effectively from the local $[Ca^{2+}]$ microdomains created after $InsP_3$-induced Ca^{2+} release [12,34].

There is also evidence that the close couplings between mitochondria and ER facilitate Ca^{2+} transfer from mitochondria to the ER via mitochondrial Na^+/Ca^{2+} exchangers releasing Ca^{2+} close to ER Ca^{2+} pumps [7–9]. In that way mitochondria would recycle the released Ca^{2+} back to the ER and would serve also to take up Ca^{2+} close to the plasma membrane and release it in inner regions of the cell close to ER-Ca^{2+} pumps. Blockade of mitochondrial Na^+/Ca^{2+} exchange with CGP37157 inhibited refilling of the ER and this effect was mimicked by mitochondrial depolarization.

Our data provide new evidence for this tight coupling of mitochondria and ER regarding Ca^{2+} homeostasis and introduce a new type of interaction, that occurring between mitochondrial Na^+/Ca^{2+} exchange and ER $InsP_3R$. It has been previously shown that inhibition of Na^+/Ca^{2+} exchange with CGP37157 increases ER-Ca^{2+} depletion induced by histamine in HeLa cells [7], a phenomenon that was attributed to inhibition of Ca^{2+} recycling between ER and mitochondria. We show here that this increased depletion occurs from the beginning of Ca^{2+} release, suggesting that an increased Ca^{2+} release in the presence of CGP37157 may also contribute to the increased depletion. This idea is reinforced by the observed changes in the oscillatory pattern induced by CGP37157. In the presence of this compound, HeLa cells reduce the frequency of the oscillations but produce larger spikes with clearly defined start and end. In contrast, in human fibroblasts, CGP37157 increases the frequency of baseline spike oscillations. Although the effects of CGP37157 in both types of cells may seem opposite, it is remarkable that in both cases the final oscillatory pattern in the presence of the inhibitor is very similar. In our opinion, both effects can be explained by an increased activation of $InsP_3R$ in the presence of CGP37157. This would generate larger spikes in HeLa cells, leading to increased ER-Ca^{2+}-depletion and feed-back Ca^{2+}-inhibition of $InsP_3R$, thus finally producing a decrease in frequency. The same mechanism, activation of $InsP_3R$, in fibroblasts would produce an increase in frequency, as these cells are either silent or show low-frequency baseline spike oscillations.

We have shown before [25] that inhibition by Ca^{2+} of $InsP_3R$ is a major factor limiting the extent of histamine-induced Ca^{2+} release. During prolonged cell stimulation with submaximal concentrations of histamine, the mean mitochondrial $[Ca^{2+}]$ keeps very low (Fig. 6), essentially at the same levels as before stimulation, although individual cells may be undergoing rapid and small Ca^{2+} spikes in parallel with the cytosolic ones, as described before for other cells [35]. Block of mitochondrial Na^+/Ca^{2+} exchanger at this point induced a small and slow increase in the mean mitochondrial $[Ca^{2+}]$, which must be due to Ca^{2+} entry into mitochondria during cytosolic oscillations, as CGP37157 produced no change in $[Ca^{2+}]_M$ in the absence of histamine

(data not shown). That increase, even though not higher than 0.5 μM $[Ca^{2+}]_M$, should be enough to activate ATP production and we show in Fig. 6 that this is the case. However, the increase in ATP was only transient, while the increase in $[Ca^{2+}]_M$ was much more prolonged, and the ATP levels increased again only after wash out of CGP37157. This phenomenon is difficult to explain but may be due to the increase in ATP consumption following the activation of ER Ca^{2+} release by CGP37157. If this hypothesis is correct, then in the presence of this compound there is both increased ATP production (by the increased $[Ca^{2+}]_M$) and increased ATP consumption (by the increased Ca^{2+} pumping). Apparently, the increase in ATP production starts earlier after addition of CGP37157, but soon equilibrates with the increased consumption, keeping ATP levels close to resting values after the initial peak. Then, when CGP37157 is removed, its effect on ATP consumption rapidly disappears, while ATP production continues increased for some time, perhaps using the energy provided by the excess NADH accumulated inside mitochondria while $[Ca^{2+}]_M$ was high. In any case, the transient increase in ATP may not account for the prolonged effect on Ca^{2+} oscillations induced by CGP37157.

The small increase in $[Ca^{2+}]_M$ induced by CGP37157 should not modify significantly the large capacity of mitochondria to take up Ca^{2+} [3]. Therefore, the main effect of CGP37157 must be that of reducing mitochondrial Ca^{2+} release and thus reduce the local $[Ca^{2+}]$ around $InsP_3R$. This would relieve in part the inhibition by Ca^{2+} of $InsP_3R$ and thus activate release. In conclusion, our data suggest that mitochondrial Na^+/Ca^{2+} exchange is involved not only in the transfer of Ca^{2+} from mitochondria to the ER but also on the inhibition of Ca^{2+} release via $InsP_3R$. Both mechanisms contribute to terminate stimulation and restore ER-Ca^{2+} levels. In addition, this new function of the mitochondrial Na^+/Ca^{2+} exchanger allows it to perform a fine tuning of the cytosolic Ca^{2+} oscillatory pattern. This effect adds to the previously shown modulation of Ca^{2+} entry [36] and K_{Ca} current [37] by this exchange system. It is interesting to note here that a functional cross-talk between the plasma membrane Na^+/Ca^{2+} exchanger and $InsP_3R$ has also been recently described [38]. Although the molecular structure of the mitochondrial Na^+/Ca^{2+} exchanger is unknown, it is tempting to speculate that both types of exchangers may interact with $InsP_3R$ by similar mechanisms.

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). JS and EH-S hold FPI and FPU fellowships, respectively, from the Spanish Ministerio de Educación y Ciencia. LV holds a fellowship from Fondo de Investigaciones Sanitarias (Spanish Ministerio de Sanidad). We thank Elena González for excellent technical assistance.

References

[1] C. Villalobos, L. Nuñez, M. Montero, A.G. García, M.T. Alonso, P. Chamero, J. Alvarez, J. García-Sancho, Redistribution of Ca^{2+} among cytosol and organella during stimulation of bovine chromaffin cells, FASEB J. 16 (2002) 343–353.

[2] D.R. Giovannucci, M.D. Hlubek, E.L. Stuenkel, Mitochondria regulate the Ca^{2+}-exocytosis relationship of bovine adrenal chromaffin cells, J. Neurosci. 19 (1999) 9261–9270.

[3] M. Montero, M.T. Alonso, E. Carnicero, I. Cuchillo-Ibañez, A. Albillos, A.G. Garcia, J. Garcia-Sancho, J. Alvarez, Chromaffin-cell stimulation triggers fast millimolar mitochondrial Ca^{2+} transients that modulate secretion, Nat. Cell Biol. 2 (2000) 57–61.

[4] J.L. Werth, S.A. Thayer, Mitochondria buffer physiological calcium loads in cultured rat dorsal root ganglion neurons, J. Neurosci. 14 (1994) 346–356.

[5] J. Herrington, Y.B. Park, D.F. Babcock, B. Hille, Dominant role of mitochondria in clearance of large Ca^{2+} loads from rat adrenal chromaffin cells, Neuron 16 (1996) 219–228.

[6] R.J. White, I.J. Reynolds, Mitochondria accumulate Ca^{2+} following intense glutamate stimulation of cultured rat forebrain neurons, J. Physiol. 498 (1997) 31–47.

[7] S. Arnaudeau, W.L. Kelley, J.V. Jr Walsh, N. Demaurex, Mitochondria recycle Ca^{2+} to the endoplasmic reticulum and prevent the depletion of neighboring endoplasmic reticulum regions, J. Biol. Chem. 276 (2001) 29430–29439.

[8] R. Malli, M. Frieden, K. Osibow, C. Zoratti, M. Mayer, N. Demaurex, W.F. Graier, Sustained Ca^{2+} transfer across mitochondria is essential for mitochondrial Ca^{2+} buffering, store-operated Ca^{2+} entry, and Ca^{2+} store refilling, J. Biol. Chem. 278 (2003) 44769–44779.

[9] R. Malli, M. Frieden, M. Trenker, W.F. Graier, The role of mitochondria for Ca^{2+} refilling of the endoplasmic reticulum, J. Biol. Chem. 280 (2005) 12114–12122.

[10] G. Hajnoczky, R. Hager, A.P. Thomas, Mitochondria suppress local feedback activation of inositol 1,4, 5-trisphosphate receptors by Ca^{2+}, J. Biol. Chem. 274 (1999) 14157–14162.

[11] G. Hajnoczky, G. Csordas, M. Madesh, P. Pacher, The machinery of local Ca^{2+} signalling between sarco-endoplasmic reticulum and mitochondria, J. Physiol. 529 (2000) 69–81.

[12] R. Rizzuto, P. Pinton, W. Carrington, F.S. Fay, K.E. Fogarty, L.M. Lifshitz, R.A. Tuft, T. Pozzan, Close contacts with the endoplasmic reticulum as determinants of mitochondrial Ca^{2+} responses, Science 280 (1998) 1763–1766.

[13] L. Filippin, P.J. Magalhaes, G. Di Benedetto, M. Colella, T. Pozzan, Stable interactions between mitochondria and endoplasmic reticulum allow rapid accumulation of calcium in a subpopulation of mitochondria, J. Biol. Chem. 278 (2003) 39224–39234.

[14] I. Bezprozvanny, J. Watras, B.E. Ehrlich, Bell shaped calcium-response curves of $Ins(1,4,5)P_3$- and calcium-gated channels from endoplasmic reticulum of cerebellum, Nature 351 (1991) 751–754.

[15] E.J. Kaftan, B.E. Ehrlich, J. Watras, Inositol 1,4,5-trisphosphate ($InsP_3$) and calcium interact to increase the dynamic range of $InsP_3$ receptor-dependent calcium signalling, J. Gen. Physiol. 110 (1997) 529–538.

[16] M. Hoth, D.C. Button, R.S. Lewis, Mitochondrial control of calcium-channel gating: a mechanism for sustained signaling and transcriptional activation in T lymphocytes, Proc. Natl. Acad. Sci. U.S.A. 97 (2000) 10607–10612.

[17] J.M. Hernandez-Guijo, V.E. Maneu-Flores, A. Ruiz-Nuno, M. Villarroya, A.G. Garcia, L. Gandia, Calcium-dependent inhibition of L, N, and P/Q Ca^{2+} channels in chromaffin cells: role of mitochondria, J. Neurosci. 21 (2001) 2553–2560.

[18] D.F. Babcock, J. Herrington, P.C. Goodwin, Y.B. Park, B. Hille, Mitochondrial participation in the intracellular Ca^{2+} network, J. Cell Biol. 136 (1997) 833–844.

[19] M. Montero, M.T. Alonso, A. Albillos, J. Garcia-Sancho, J. Alvarez, Mitochondrial Ca^{2+}-induced Ca^{2+} release mediated by the Ca^{2+} uniporter, Mol. Biol. Cell. 12 (2001) 63–71.
[20] T.J. Collins, P. Lipp, M.J. Berridge, W. Li, M.D. Bootman, Inositol 1,4,5-trisphosphate-induced Ca^{2+} release is inhibited by mitochondrial depolarization, Biochem. J. 347 (2000) 593–600.
[21] M. Montero, M. Brini, R. Marsault, J. Alvarez, R. Sitia, T. Pozzan, R. Rizzuto, Monitoring dynamic changes in free Ca^{2+} concentration in the endoplasmic reticulum of intact cells, EMBO J. 14 (1995) 5467–5475.
[22] M. Montero, C.D. Lobaton, A. Moreno, J. Alvarez, A novel regulatory mechanism of the mitochondrial Ca^{2+} uniporter revealed by the p38 mitogen-activated protein kinase inhibitor SB202190, FASEB J. 16 (2002) 1955–1957.
[23] G. Grynkiewicz, M. Poenie, R.Y. Tsien, A new generation of Ca^{2+} indicators with greatly improved fluorescence properties, J. Biol. Chem. 260 (1985) 3440–3450.
[24] P. Bernardi, Mitochondrial transport of cations: channels, exchangers and permeability transition, Physiol. Rev. 79 (1999) 1127–1155.
[25] M. Montero, M.J. Barrero, J. Alvarez, [Ca^{2+}] microdomains control agonist-induced Ca^{2+} release in intact HeLa cells, FASEB J. 11 (1997) 881–885.
[26] K.T. Baron, S.A. Thayer, CGP37157 modulates mitochondrial Ca^{2+} homeostasis in cultured rat dorsal root ganglion neurons, Eur. J. Pharmacol. 340 (1997) 295–300.
[27] L.S. Jouaville, P. Pinton, C. Bastianutto, G.A. Rutter, R. Rizzuto, Regulation of mitochondrial ATP synthesis by calcium: evidence for a long-term metabolic priming, Proc. Natl. Acad. Sci. U.S.A. 96 (1999) 13807–13812.
[28] D.O. Mak, S. McBride, J.K. Foskett, ATP regulation of recombinant type 3 inositol 1,4,5-trisphosphate receptor gating, J. Gen. Physiol. 117 (2001) 431–434.
[29] T. Miyakawa, A. Mizushima, K. Hirose, T. Yamazawa, I. Bezprozvanny, T. Kurosaki, M. Iino, Ca^{2+}-sensor region of IP_3 receptor controls intracellular Ca^{2+} signaling, EMBO J. 20 (2001) 1674–1680.
[30] J.W. Putney Jr., G.S. Bird, The inositol phosphate-calcium signaling system in nonexcitable cells, Endocr. Rev. 14 (1993) 610–631.
[31] C. Fewtrell, Ca^{2+} oscillations in non-excitable cells, Annu. Rev. Physiol. 55 (1993) 427–454.
[32] M.J. Berridge, G. Dupont, Spatial and temporal signalling by calcium, Curr. Opin. Cell. Biol. 6 (1994) 267–274.
[33] M. Hattori, A.Z. Suzuki, T. Higo, H. Miyauchi, T. Michikawa, T. Nakamura, T. Inoue, K. Mikoshiba, Distinct roles of inositol 1,4,5-trisphosphate receptor types 1 and 3 in Ca^{2+} signaling, J. Biol. Chem. 279 (2004) 11967–11975.
[34] G. Csordas, A.P. Thomas, G. Hajnoczky, Quasi-synaptic calcium signal transmission between endoplasmic reticulum and mitochondria, EMBO J. 18 (1999) 96–108.
[35] C. Villalobos, L. Nuñez, P. Chamero, M.T. Alonso, J. Garcia-Sancho, J. Mitochondrial, [Ca^{2+}] oscillations driven by local high [Ca^{2+}] domains generated by spontaneous electric activity, J. Biol. Chem. 276 (2001) 40293–40297.
[36] B. Lee, P.D. Miles, L. Vargas, P. Luan, S. Glasco, Y. Kushnareva, E.S. Kornbrust, K.A. Grako, C.B. Wollheim, P. Maechler, J.M. Olefsky, C.M. Anderson, Inhibition of mitochondrial Na^{+}-Ca^{2+} exchanger increases mitochondrial metabolism and potentiates glucose-stimulated insulin secretion in rat pancreatic islets, Diabetes 52 (2003) 965–973.
[37] S.Y. Cheranov, J.H. Jaggar, Mitochondrial modulation of Ca^{2+} sparks and transient K_{Ca} currents in smooth muscle cells of rat cerebral arteries, J. Physiol. 556 (2004) 755–771.
[38] L.M. Solis-Garrido, A.J. Pintado, E. Andres-Mateos, M. Figueroa, C. Matute, C.C. Montiel, Cross-talk between native plasmalemmal Na^{+}/Ca^{2+} exchanger and inositol 1,4,5-trisphosphate-sensitive Ca^{2+} internal store in *Xenopus* oocytes, J. Biol. Chem. 279 (2004) 52414–52424.