Effect of inositol 1,4,5-trisphosphate receptor stimulation on mitochondrial [Ca^{2+}] and secretion in chromaffin cells

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Ca^{2+} uptake by mitochondria is a potentially important buffering system able to control cytosolic [Ca^{2+}]. In chromaffin cells, we have shown previously that stimulation of either Ca^{2+} entry or Ca^{2+} release via ryanodine receptors triggers large increases in mitochondrial [Ca^{2+}] ($[Ca^{2+}]_M$) approaching the millimolar range, whose blockade dramatically enhances catecholamine secretion [Montero, Alonso, Carnicero, Cuchillo-Ibañez, Albillos, Garcia, Carcia-Sancho and Alvarez (2000) Nat. Cell Biol. **2**, 57–61]. In the present study, we have studied the effect of stimulation of inositol 1,4,5-trisphosphate ($InsP_3$) receptors using histamine. We find that histamine produces a heterogeneous increase in $[Ca^{2+}]_M$, reaching peak levels at approx. 1 μM in 70 % of the mitochondrial space to several hundred micromolar in 2–3 % of mitochondria. Intermediate levels were found in the rest of the mitochondrial space. Single-cell imaging experiments with aequorin showed that the heterogeneity had both an intercellular and a subcellular origin. Those mitochondria responding to histamine with increases in $[Ca^{2+}]_M$ much greater than 1 μM (30 %) were the same as those that also responded with large increases in $[Ca^{2+}]_M$ following stimulation with either high-K^+ medium or caffeine. Blocking mitochondrial Ca^{2+} uptake with protonophores or mitochondrial inhibitors also enhanced catecholamine secretion induced by histamine. These results suggest that some $InsP_3$ receptors tightly co-localize with ryanodine receptors and voltage-dependent Ca^{2+} channels in defined subplasmalemmal functional units designed to control secretion induced by different stimuli.

Key words: aequorin, catecholamine secretion, histamine.

INTRODUCTION

In recent years, growing evidence suggests that mitochondria strategically placed in the vicinity of Ca^{2+} entry or release sites play an important role in local Ca^{2+} signalling. Structural studies have shown in different cell types that some mitochondria are placed in close proximity to the endoplasmic reticulum (ER) [1–5] or the plasma membrane [5–7]. Consistently, functional studies have also indicated that mitochondria take up large amounts of Ca^{2+} after ER Ca^{2+} release [5,7–10] or Ca^{2+} entry from the extracellular medium [5–7,11–16]. Because of the low Ca^{2+}-affinity of the mitochondrial Ca^{2+} import system (the Ca^{2+} uniporter), this large Ca^{2+} uptake can only happen if some mitochondria are located at short distances from the mouth of the Ca^{2+} channels. These mitochondria would sense local [Ca^{2+}] much greater than the mean cytosolic [Ca^{2+}] ($[Ca^{2+}]_c$), thus taking up Ca^{2+} much faster (for a review see [17]).

The best evidence for this hypothesis has been obtained using targeted aequorin, and taking advantage of the property of aequorin of being selectively consumed in high-[Ca^{2+}] environments. In HeLa cells, stimulation with inositol 1,4,5-trisphosphate ($InsP_3$)-producing agonists induced a peak of mitochondrial [Ca^{2+}] ($[Ca^{2+}]_M$) that consumed approx. 30 % of the aequorin in a few seconds, but further stimulation induced much smaller $[Ca^{2+}]_M$ responses, although similar $[Ca^{2+}]_c$ peaks were obtained [9]. These findings suggested that ER Ca^{2+} release induced a large increase in [Ca^{2+}] in only approx. 30 % of the mitochondria and, in particular, those placed closer to the ER. This idea was confirmed by further studies in permeabilized cells showing that the buffered [Ca^{2+}] required to mimic the mitochondrial Ca^{2+} uptake induced by histamine was much higher than that measured in the cytoplasm after histamine stimulation. Moreover, the whole mitochondrial space took up Ca^{2+} homogeneously during perfusion with [Ca^{2+}] buffers [8,9].

We have recently attained similar conclusions working in chromaffin cells [7], but, in this case, approx. 50 % of the mitochondria were associated with voltage-dependent Ca^{2+} channels in the plasma membrane. Most of these mitochondria were also coupled to ER Ca^{2+} release via ryanodine receptors. A larger increase in $[Ca^{2+}]_M$ after cell depolarization in mitochondria close to the plasma membrane has been also confirmed by structural studies obtained with X-ray microanalysis [6]. Given that Ca^{2+} uptake by these mitochondria also appeared to control catecholamine secretion [7,18], we developed the concept of functional units to account for the close coupling observed among plasma-membrane Ca^{2+} channels, ER ryanodine receptors, mitochondria and secretory vesicles [7]. In the present study, we have investigated the effects of $InsP_3$-receptor stimulation and their relationship with the above-mentioned functional units. Our results show that $InsP_3$ receptors are also present in the functional units and their stimulation produces large increases in $[Ca^{2+}]_M$ in a small subpopulation of mitochondria. Moreover,

Abbreviations used: AEQ1, wild-type aequorin reconstituted with native coelenterazine; AEQ2, wild-type aequorin reconstituted with coelenterazine n; AEQ3, mutated aequorin reconstituted with coelenterazine n; $[Ca^{2+}]_c$, cytosolic [Ca^{2+}]; $[Ca^{2+}]_M$, mitochondrial [Ca^{2+}]; ER, endoplasmic reticulum; $[Ca^{2+}]_{ER}$, ER [Ca^{2+}]; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; $InsP_3$, inositol 1,4,5-trisphosphate.

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abolition of mitochondrial Ca^{2+} uptake strongly enhanced catecholamine secretion induced by $InsP_3$-generating agonists.

EXPERIMENTAL

Cell culture and expression of aequorin

Bovine adrenal medulla chromaffin cells were obtained as described previously [19] and were used within 1–3 days. Aequorins targeted to mitochondria and cytosol [20,21] were gifts from Professor Tullio Pozzan, Department of Medical Sciences, University of Padova, Padova, Italy. Mutated mitochondrial aequorin ($Asp^{119} \rightarrow Ala$) was obtained by replacing in-frame the wild-type aequorin with the mutated aequorin DNA [22]. Preparation of mutated aequorin targeted to the ER has been described previously [22]. Expression in chromaffin cells was achieved by infecting the cells with a defective herpes simplex virus type 1 containing the gene for each type of targeted aequorin. Virus packaging and titreing have been described previously [23]. Chromaffin cell cultures (5×10^5 cells/0.5 ml) were routinely infected 12–24 h prior to measurements with 2×10^3 infectious virus units.

$[Ca^{2+}]_M$, $[Ca^{2+}]_c$ and ER $[Ca^{2+}]$ ($[Ca^{2+}]_{ER}$) measurements in cell populations

For aequorin reconstitution, cells expressing wild-type aequorin targeted to the cytosol were incubated for 1–2 h at 22 °C with 1 μM of wild-type coelenterazine in standard incubation medium [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 expressing wild-type aequorin targeted to the mitochondria were incubated for 1–2 h at 22 °C with either 1 μM of wild-type coelenterazine (AEQ1, possessing high Ca^{2+}-affinity) or 1 μM of coelenterazine n (AEQ2, possessing intermediate Ca^{2+}-affinity). Cells expressing mutated aequorin targeted to the mitochondria were incubated for 1–2 h at room temperature with 1 μM of coelenterazine n (AEQ3; possessing low Ca^{2+}-affinity). Cells were then placed in the perfusion chamber of a purpose-built thermostatic luminometer at 37 °C. In the case of aequorin targeted to the ER, Ca^{2+} depletion of the ER was required prior to reconstitution. In this case, cells were incubated for 10 min in medium containing 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 0.5 mM EGTA, 10 μM 2,5-di(tert-butyl)hydroquinone (an inhibitor of ER Ca^{2+}-ATPase), 10 mM glucose and 10 mM Hepes (pH 7.4). After washing once with the above medium, cells were placed in the same medium in the presence of 1 μM coelenterazine n for 1–2 h. Before starting the experiment, cells were placed in the perfusion chamber of the luminometer and perfused with the same medium without 2,5-di(tert-butyl)hydroquinone for at least 5 min. Experiments with ER-targeted aequorin were performed at 22 °C to reduce the rate of aequorin consumption.

In order to calibrate the data obtained in terms of $[Ca^{2+}]_M$, the total amount of luminescence emitted by the sample is required. Thus at the end of every experiment it is essential to perfuse lysis solution containing detergent (100 μM digitonin) and excess Ca^{2+} (10 mM) to measure all the remaining aequorin luminescence. To transform luminescence data into $[Ca^{2+}]$, a computer program was used to subtract the background and calculate the fractions L/L_{max} at every point along the experiment [where L is the luminescence value at each point (minus the background) and L_{max} is the integral of luminescence (minus the background) from that point to the end of the experiment]. L/L_{max} values were then transformed into $[Ca^{2+}]$ values using a mathematical algorithm [21]. In some experiments, most of the luminescence was assumed to come from a subcompartment of the whole mitochondrial space (see below). In these cases, calibration of the real $[Ca^{2+}]$ reached in the subcompartment was achieved by using a value of L_{max} corresponding to approximately the total luminescence coming from that subcompartment. Calibration was performed using the calibration curves corresponding to each aequorin type and temperature [7,24,25]. Aequorin consumption was calculated as the integral of the luminescence measured during the course of the experiment and normalized as a percentage.

Measurements of single-cell $[Ca^{2+}]_M$

For bioluminescence imaging [26,27], cells were placed into a thermostatic perfusion chamber at 37 °C under a Zeiss Axiovert S100 TV microscope (Fluar 40× oil, 1.3 numeric aperture objective) and perfused at 5–10 ml/min with the test solutions prewarmed to 37 °C. At the end of each experiment, cells were permeabilized with 100 μM digitonin in 10 mM $CaCl_2$ to release all the residual aequorin counts. Images were taken with a Hamamatsu VIM photon-counting camera handled with an Argus-20 image processor and integrated for 10 s periods. Photons/cell in each image were quantified using the Hamamatsu Aquacosmos software. Total counts per cell ranged from between 10^3 and 10^5 and noise was 1 ± 1 counts per second per typical cell area (2000 pixels; mean ± S.D.). Data were first quantified as rates of photoluminescence emission/total counts per second remaining at each time point and divided by the integration period (L/L_{TOTAL}, in s^{-1}). Calibrations of $[Ca^{2+}]$ were performed using the calibration curve described previously [7,21]. A transmission image was also taken at the beginning of each experiment.

Measurements of single-cell $[Ca^{2+}]_c$

Single-cell measurements of $[Ca^{2+}]_c$ were performed in cells loaded with fura 2 (2 μM; 60 min at 25 °C) as described previously [28]. Cells were epi-illuminated alternatively at 340 and 380 nm and light emitted above 520 nm was recorded by an extended ISIS-M camera (Photonic Science, Robertbridge, East Sussex, U.K.) and analysed using an Applied Imaging Magical image processor (Sunderland, U.K.). Eight frames excited at every wavelength were averaged by hardware with a time resolution of 1.7 s for each pair of images. All the experiments were performed at 37 °C.

On-line measurements of catecholamine release from populations of bovine chromaffin cells

Cells were stimulated to secrete catecholamines with short pulses (2 s) of Krebs–Hepes solution [144 mM NaCl, 5.9 mM KCl, 2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 11 mM glucose and 10 mM Hepes (pH 7.4)] containing histamine. Following stimulation, cells were carefully scraped off the bottom of the Petri dish with a rubber policeman and centrifuged at 120 ***g*** for 10 min at 22 °C. The cell pellet was resuspended in 200 μl of Krebs–Hepes solution. Cells were introduced in a jacketed microchamber for superfusion at 37 °C at a rate of 2 ml/min. The liquid flowing from the superfusion chamber passed through an electrochemical detector (model 641 VA; Metrohm AG, Herisau, Switzerland) placed at the outlet of the microchamber. This enabled the on-line monitoring, in the amperometric mode, of the amount of catecholamines secreted [29].

RESULTS

Stimulation of bovine chromaffin cells with histamine produces a fast Ca^{2+} release from the ER, via $InsP_3$ receptors, that can be elicited repetitively with little signs of desensitization [30]. Figure 1

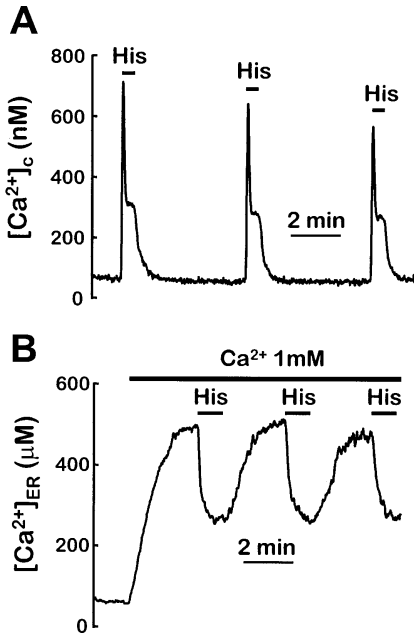

Figure 1 Effect of histamine on $[Ca^{2+}]_c$ and $[Ca^{2+}]_{ER}$ measured with targeted aequorin

(**A**) Chromaffin cells expressing cytosolic wild-type aequorin were reconstituted with native coelenterazine for 1–2 h. Three consecutive 30 s pulses of 10 μM histamine (His) were then applied as indicated. (**B**) Chromaffin cells expressing ER-targeted mutated aequorin were depleted of Ca^{2+} and reconstituted with coelenterazine n for 1–2 h. Cells were then perfused with medium containing 1 mM Ca^{2+}, as indicated, to refill the ER with Ca^{2+}. When $[Ca^{2+}]_{ER}$ reached steady-state, three consecutive 1 min pulses of 10 μM histamine were applied as indicated. Results are from representative experiments performed three times with similar results.

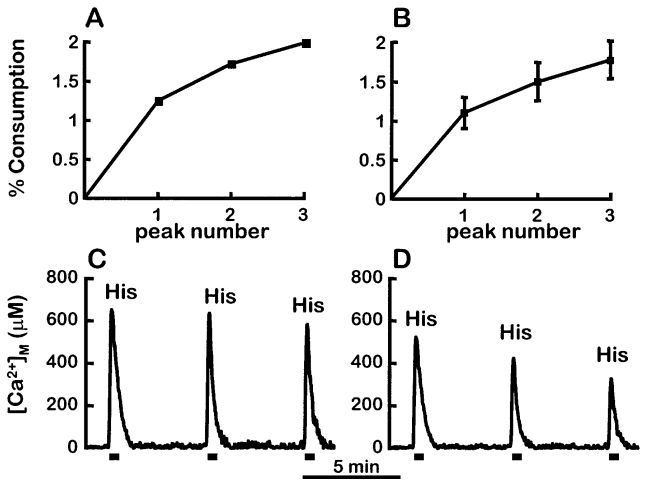

Figure 3 Calculation of $[Ca^{2+}]_M$ changes in a small highly responsive mitochondrial compartment

(**A**) Cumulative aequorin consumption from the three $[Ca^{2+}]_M$ peaks obtained during the stimulations with histamine applied in the experiment shown in Figure 2(C). (**B**) Cumulative aequorin consumption from the three $[Ca^{2+}]_M$ peaks obtained from five experiments similar to that shown in Figure 2(C). Results are means ± S.D. (**C** and **D**) Recalibration of the experiment shown in Figure 2(C) considering that $[Ca^{2+}]_M$ changes occurred only in 2.5% (**C**) or 3% (**D**) of the total mitochondrial space. Additions of histamine (His) are indicated by black bars below the $[Ca^{2+}]_M$ traces.

shows the responses of both $[Ca^{2+}]_c$ (Figure 1A) and $[Ca^{2+}]_{ER}$ (Figure 1B) to a series of three pulses of histamine. Measurements were performed using high-Ca^{2+}-affinity aequorin (AEQ1) specifically targeted to the cytosol, or low-Ca^{2+}-affinity aequorin (AEQ3) specifically targeted to the ER. It can be appreciated that both the kinetics and the amplitude of the consecutive peaks of $[Ca^{2+}]_c$ and decreases in $[Ca^{2+}]_{ER}$ were similar.

To study the effect of histamine on $[Ca^{2+}]_M$, three aequorin probes with different Ca^{2+} affinities were used as reported previously [7]. AEQ1 (high Ca^{2+}-affinity) and AEQ2 (intermediate Ca^{2+}-affinity) correspond to mitochondrially targeted native aequorin reconstituted with either wild-type coelenterazine or the semi-synthetic derivative coelenterazine n respectively.

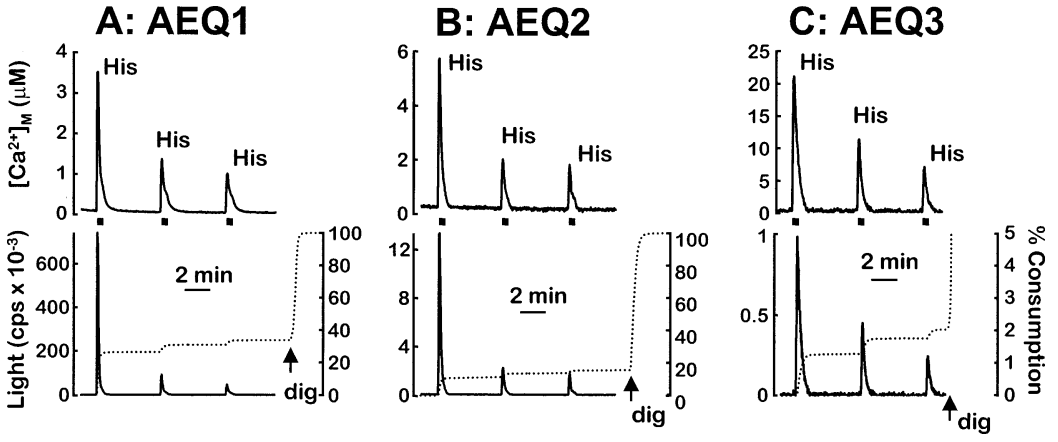

Figure 2 Effect of histamine on $[Ca^{2+}]_M$ and aequorin consumption using targeted aequorins with different Ca^{2+} affinities

Experiments were carried out using chromaffin cells expressing aequorin targeted to the mitochondria with (**A**) high (AEQ1), (**B**) intermediate (AEQ2) or (**C**) low (AEQ3) Ca^{2+}-affinities as described in the Experimental section. In each experiment, three consecutive 30 s pulses of 10 μM histamine (His) were applied (indicated by black bars below the $[Ca^{2+}]_M$ traces). The upper part of each panel shows the calibrated $[Ca^{2+}]_M$ trace. The lower part shows the crude luminescence record (solid line) and the accumulative aequorin consumption (dotted line) for the same experiment in counts per second (cps). Aequorin consumption is expressed as the percentage of the total luminescence emitted by the sample, including that released by the addition of 100 μM digitonin (dig) in 10 mM Ca^{2+} medium at the end of every experiment. Results are from representative experiments performed 5–12 times with similar results.

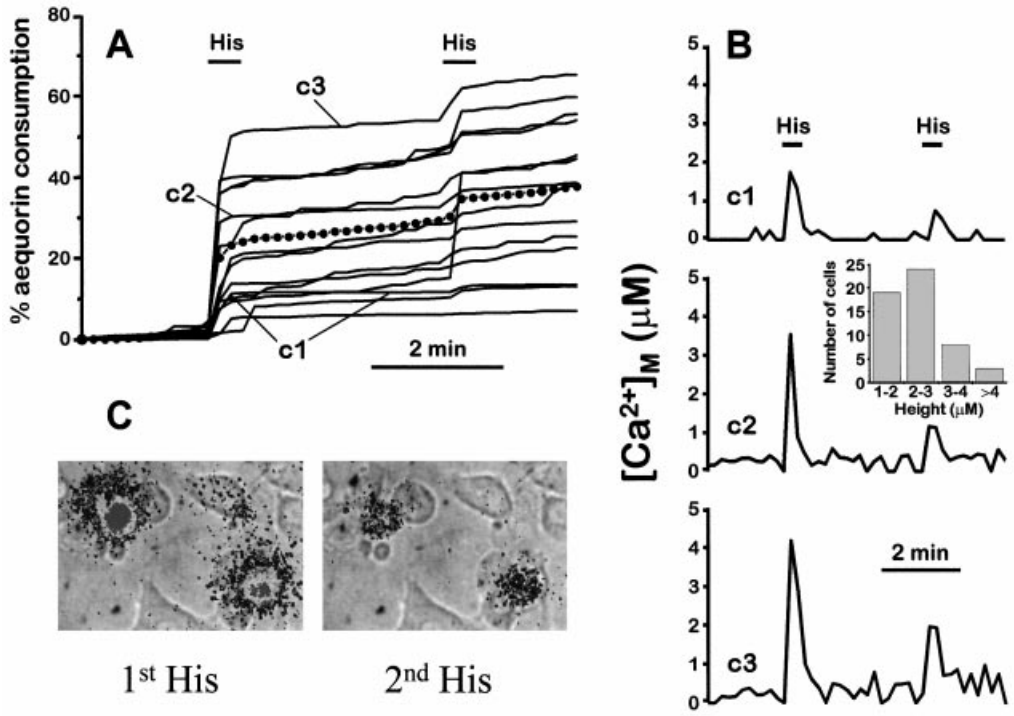

Figure 4 Single-cell imaging of chromaffin cells expressing aequorin

Chromaffin cells expressing mitochondrially targeted high-Ca^{2+}-affinity aequorin (AEQ1) were stimulated twice with 10 μM histamine (His) as indicated. (**A**) The aequorin consumption traces of 14 single cells obtained from two different experiments with the same protocol. The dotted line shows the mean of all traces, and the traces labelled c1, c2, and c3 correspond to the same cells whose $[Ca^{2+}]_M$ records are shown in (**B**). (**B**) Three representative single-cell $[Ca^{2+}]_M$ responses to the two consecutive histamine stimulations. The inset shows the distribution of the $[Ca^{2+}]_M$ peak heights in 54 cells stimulated with 10 μM histamine in five different experiments. (**C**) Two bioluminescent images acquired during the first (1st His) and second (2nd His) pulses of histamine and superimposed over the bright-field image. It should be noted that the photonic emissions acquired during the first pulse were more abundant than during the second one. Image sizes, 88×61 μm.

AEQ3 (low Ca^{2+}-affinity) corresponds to mitochondrially targeted mutated aequorin reconstituted with coelenterazine n, and is the same type of aequorin used above to measure $[Ca^{2+}]_{ER}$. Figure 2 shows the effect of three consecutive additions of histamine on the response of chromaffin cells expressing each type of mitochondrially targeted aequorin. The upper part of each panel shows the calibrated $[Ca^{2+}]_M$ responses and the lower part the crude luminescence data (solid lines) and the time course of aequorin consumption during the experiments (dotted lines). Figures 2(A), 2(B) and 2(C) show the data obtained using cells expressing mitochondrially targeted AEQ1, AEQ2 and AEQ3 respectively. The initial $[Ca^{2+}]_M$ peak was larger than the others in all the cases, and its magnitude increased considerably when the aequorin with lower affinity (AEQ3) was used. The first $[Ca^{2+}]_M$ peak reached 4.1 ± 0.2 μM (mean $\pm$ S.E.M., $n = 5$) for cells expressing AEQ1, 5.0 ± 0.2 μM (mean $\pm$ S.E.M., $n = 6$) for cells expressing AEQ2 and 19.1 ± 0.7 μM (mean $\pm$ S.E.M., $n = 12$) for cells expressing AEQ3.

The only explanation for these discrepancies in the calibrated values obtained with the different aequorins is that the mitochondrial Ca^{2+} signal during stimulation is high enough to saturate AEQ1 and AEQ2. When aequorin becomes saturated with Ca^{2+}, the rate of light emission is no longer proportional to the $[Ca^{2+}]$, and becomes so fast that most of the aequorin molecules emit their photons within a few seconds. This should lead to a complete consumption of the aequorin exposed to such high $[Ca^{2+}]$. However, the aequorin-consumption traces (dotted lines) show that only a relatively small percentage of the total AEQ1 or AEQ2 was consumed after the first histamine stimulation. Aequorin consumption after the first histamine addition was 30 ± 1 % (mean $\pm$ S.E.M., $n = 5$) in cells expressing AEQ1 and 9.6 ± 0.3 % (mean $\pm$ S.E.M., $n = 6$) in cells expressing AEQ2. This suggests that saturation of AEQ1 or AEQ2 may occur only in a small mitochondrial subcompartment, leading to nearly complete consumption of the aequorin enclosed within that compartment. If this were correct, subsequent stimulations with histamine that produce similar responses in terms of $[Ca^{2+}]_c$ or $[Ca^{2+}]_{ER}$ (see Figure 1) should produce a much smaller luminescence response from mitochondrial aequorin. This was the case, because the first luminescence peak induced by histamine was much larger than the second one (8.4 ± 0.3-fold for AEQ1 and 4.9 ± 0.4-fold for AEQ2; values are means $\pm$ S.E.M., with $n = 4$ for AEQ1 and $n = 5$ for AEQ2). On the contrary, the second and third peaks were much more similar both in terms of consumption and calibrated $[Ca^{2+}]_M$ values, suggesting that saturation of the aequorin signal occurred mainly or only in the first stimulus. This indicates that the percentage of aequorin consumption during the first stimulation with histamine should correspond approximately to the size of the mitochondrial space in which saturation of the probe with Ca^{2+} has taken place. Therefore stimulation with histamine induces a heterogeneous increase in $[Ca^{2+}]_M$ that saturates AEQ1 ($[Ca^{2+}]_M \geqslant 10$ μM) in approx. 30 % of the mitochondrial space, whereas the same stimulus saturates AEQ2 ($[Ca^{2+}]_M \geqslant 40$ μM) in only approx. 10 % of that space.

Regarding the data obtained with AEQ3, consumption after the first histamine stimulation was much smaller (1.1 ± 0.2 %; mean $\pm$ S.E.M., $n = 5$; notice the different scale in Figure 2C).

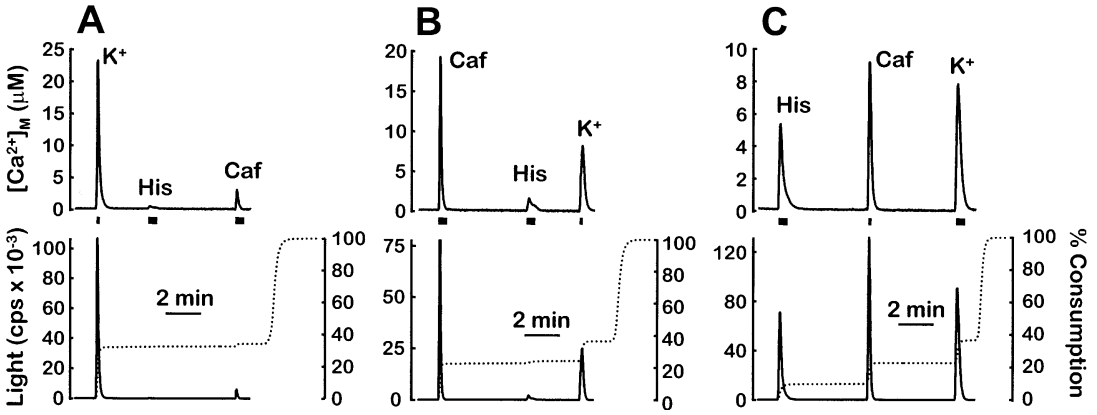

Figure 5 Overlap amongst the mitochondrial spaces responding with large $[Ca^{2+}]_M$ peaks to histamine and to high K^+ or caffeine as determined using AEQ2

Chromaffin cells expressing intermediate-Ca^{2+}-affinity aequorin (AEQ2) were stimulated with 30 s pulses of standard incubation medium containing 10 μM histamine (His), 10 s pulses of high-K^+ medium (K^+) [80 mM NaCl, 70 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, 10 mM Hepes (pH 7.4)] and 30 s pulses of standard incubation medium containing 50 mM caffeine (Caf) as indicated. (**A**) Cells were treated with high-K^+ medium prior to histamine and caffeine; (**B**) cells were treated with caffeine prior to histamine and high-K^+ medium; and (**C**) cells were treated with histamine prior to caffeine and high-K^+ medium. Details of the upper and lower panels are given in Figure 2. Results are from representative experiments performed six times with similar results.

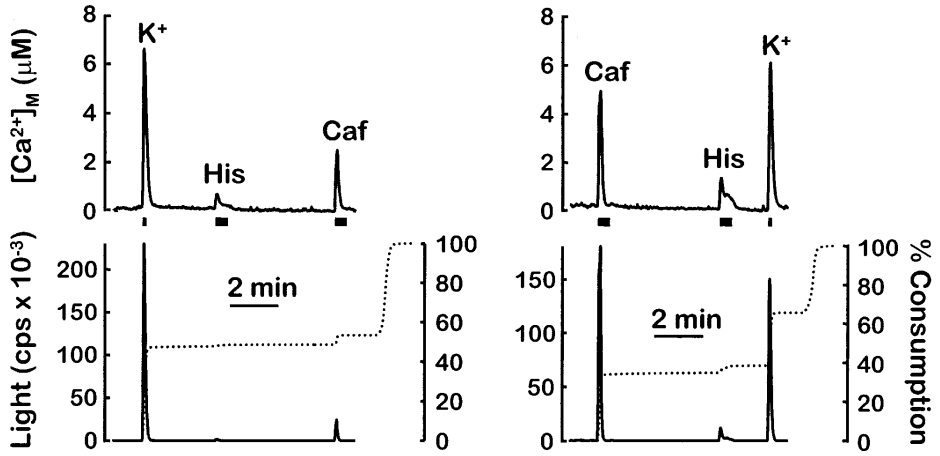

Figure 6 Overlap amongst the mitochondrial spaces responding with large $[Ca^{2+}]_M$ peaks to histamine and to high K^+ or caffeine as determined using AEQ1

Chromaffin cells expressing high-Ca^{2+}-affinity aequorin (AEQ1) were stimulated with 30 s pulses of standard incubation medium containing 10 μM histamine (His), 10 s pulses of high-K^+ medium (K^+) [80 mM NaCl, 70 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, 10 mM Hepes (pH 7.4)] and 30 s pulses of standard incubation medium containing 50 mM caffeine (Caf). Left-hand panel: cells were treated with high-K^+ medium prior to histamine and caffeine. Right-hand panel: cells were treated with caffeine prior to histamine and high-K^+ medium. Details of the upper and lower panels are given in Figure 2. Results are from representative experiments performed four times with similar results.

However, both the height of the luminescence peaks and the consumption decreased rapidly in the second and third consecutive peaks. Figure 3(A) shows the accumulated AEQ3 consumption in the experiment from Figure 2(C), which was comparable with the mean data obtained from five similar experiments (Figure 3B). These accumulated aequorin consumption patterns could be fitted by exponential or sigmoidal curves (the fitting procedure did not significantly modify the final result) that gave extrapolated values for the maximum expected consumption after a long series of peaks to be approx. 2–2.5% (results not shown). This suggests that histamine induces a very large increase in $[Ca^{2+}]_M$, enough to rapidly consume AEQ3, but occurring in only a small mitochondrial subcompartment (approx. 2–2.5%). To estimate the size of these peaks, we can recalculate the $[Ca^{2+}]_M$ data from Figure 2(C) assuming that the mitochondrial space where the $[Ca^{2+}]$ changes are taking place is much smaller than the total. Figure 3(C) shows the $[Ca^{2+}]_M$ traces obtained from the experiment in Figure 2(C) recalculated by assuming that $[Ca^{2+}]_M$ changes occur in only 2.5% of the total mitochondrial space. To perform these calculations, the total luminescence of the experiment (L_{max}, see the Experimental section) was reduced to the desired percentage, so that the L/L_{max} ratios reflect the real value in the subcompartment. We can see here that consecutive additions of histamine evoke repetitive similar $[Ca^{2+}]_M$ peaks at approx. 500 μM in that small subcompartment. Obviously, the exact size of that high Ca^{2+} subcompartment is difficult to assess. Increasing the size of the subcompartment reduced the magnitude of the calculated $[Ca^{2+}]$ peaks. For example, Figure 3(D) shows the result of using a total space of 3% for the calculations. The peaks are now somewhat smaller, but they keep within the 300–500 μM range. In fact, increasing the percentage to even 5% still gave calibrated $[Ca^{2+}]$ peaks of 300 μM, 200 μM and 130 μM. The values obtained using percentages of 2.5–3%, however, are more

consistent with the $[Ca^{2+}]_c$ and $[Ca^{2+}]_{ER}$ data in Figure 1, which showed similar repetitive effects of histamine in the three consecutive peaks.

The results described above indicate that, after stimulation of chromaffin cells with histamine, we can distinguish a series of different mitochondrial compartments with different $[Ca^{2+}]$. Approx. 70 % of the mitochondria responded with very small increases in $[Ca^{2+}]_M$ of approx. 1 μM (Figure 2A). The other 30 % responded with higher $[Ca^{2+}]_M$ peaks, but this response was not homogeneous. Approx. 20 % underwent $[Ca^{2+}]_M$ increases to approx. 10 μM (saturation of AEQ1, but not of AEQ2), 2–3 % to several hundred micromolar (measured by AEQ3) and the rest reached intermediate levels (approx. 40 μM; AEQ2 saturation, but little effect on AEQ3). This heterogeneity could have either an intercellular or a subcellular origin. Single-cell $[Ca^{2+}]_c$ imaging experiments showed that most of the cells responded to histamine, and the response was fairly homogeneous. Only 20 % of the cells gave $[Ca^{2+}]_c$ peaks below 200 nM, and the mean $[Ca^{2+}]_c$ peak in the rest of the cells was 730 ± 50 nM (mean $\pm$ S.E.M., $n = 45$). Nevertheless, we decided to investigate directly the presence of intercellular and/or subcellular $[Ca^{2+}]_M$ heterogeneity by performing single-cell aequorin imaging experiments using chromaffin cells expressing AEQ1. Figure 4(A) shows the aequorin consumption traces from a series of single cells stimulated with histamine. In contrast with the $[Ca^{2+}]_c$ peaks, there was a large variability in the percentage of aequorin consumed by the first histamine stimulation, from less than 10 % to more than 50 %. Three representative single-cell $[Ca^{2+}]_M$ traces are also shown (Figure 4B) to highlight the variability in terms of both height and width of the $[Ca^{2+}]_M$ peaks. The distribution of peak heights shown in the inset gives a better idea of the wide distribution of $[Ca^{2+}]_M$ responses. A second addition of histamine produced only a minor $[Ca^{2+}]_M$ peak in each case, in spite of the fact that repetitive histamine stimulation produced similar $[Ca^{2+}]_c$ peaks. These results clearly suggest that heterogeneity has both subcellular and intercellular components.

Another relevant question is whether or not these mitochondrial spaces are somehow related to those activated by high K^+ depolarization or caffeine. We have shown previously [7] that activation of Ca^{2+} entry with high K^+ produces a large $[Ca^{2+}]_M$ peak of approx. 500 μM in 50 % of mitochondria, and stimulation of ryanodine receptors with caffeine produces a similarly large $[Ca^{2+}]_M$ peak in 30 % of mitochondria. In both cases, full saturation of AEQ2 was reached in those mitochondria, and the real $[Ca^{2+}]_M$ values could only be measured using AEQ3. High K^+ stimulation abolished the subsequent $[Ca^{2+}]_M$ response induced by caffeine, suggesting that both mitochondrial spaces overlapped completely (30 % of mitochondria responded to both, and an additional 20 % only to high K^+). These experiments suggested that plasma-membrane Ca^{2+} channels and ryanodine receptors were co-localized with mitochondria constituting defined functional units [7]. In the present study, we have performed similar experiments using histamine, caffeine and high K^+. Figure 5 shows the effect of the consecutive addition of these stimuli on $[Ca^{2+}]_M$ and aequorin consumption in cells expressing AEQ2. Both high K^+ (Figure 5A) and caffeine stimulation (Figure 5B) abolished the $[Ca^{2+}]_M$ response to histamine, particularly in terms of aequorin consumption. Figure 5(C) shows a control experiment carried out in parallel where histamine was added first and partially reduced the subsequent response to caffeine. These results suggest that the mitochondria that respond with high $[Ca^{2+}]_M$ peaks to histamine (AEQ2 saturation: approx. 10 % of the mitochondrial space, including 2–3 % that produced peaks of several hundred micromolar and 7–8 % responding with peaks of approx. 40 μM) also respond with high $[Ca^{2+}]_M$ peaks to the other stimuli. Of course, the $[Ca^{2+}]_M$ response to histamine was much smaller than that induced by high K^+ or caffeine both in the magnitude of the $[Ca^{2+}]_M$ peaks and in the percentage of mitochondrial space involved.

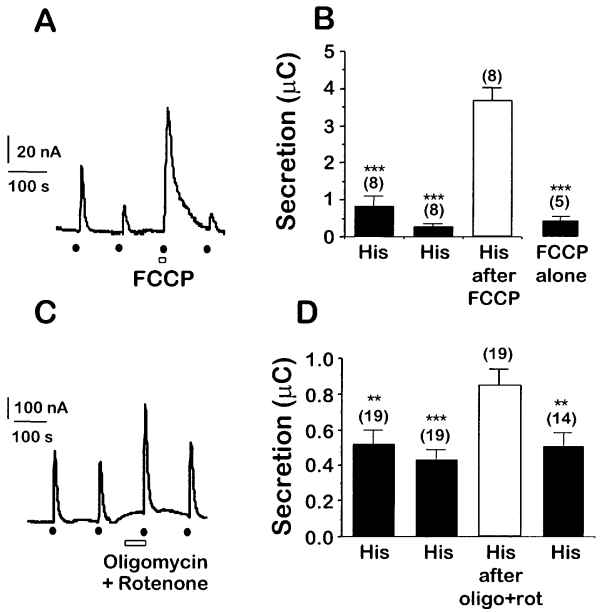

Figure 7 Effect of blocking mitochondrial Ca^{2+} uptake on histamine-induced catecholamine secretion

(**A** and **B**) Cells were stimulated with two 2 s pulses of a solution containing 10 μM histamine (closed circle). FCCP (20 μM) was perfused for 5 s before the third histamine pulse as indicated. (**A**) A trace from a representative experiment showing the two control histamine-induced peaks followed by one histamine-induced peak after FCCP treatment. (**B**) Mean secretion obtained in eight similar experiments to that shown in (**A**). The secretion obtained after treatment with FCCP alone is also shown. (**C** and **D**) Cells were stimulated with two 2 s pulses of a standard incubation medium containing 10 μM histamine (closed circle). Oligomycin (3 μM) and rotenone (4 μM) were perfused for 90 s before the histamine pulse as indicated. (**C**) A trace from a representative experiment showing the two control histamine-induced peaks followed by one histamine-induced peak after treatment with oligomycin and rotenone (oligo + rot). (**D**) Mean secretion obtained from 19 similar experiments to that shown in (**C**). Error bars in (**B**) and (**D**) are expressed as S.E.M.s of the area under the corresponding spike (μC) in experiments similar to those in (**A**) and (**C**). $^{**}P < 0.05$ between histamine treatments and histamine after oligomycin and rotenone; $^{***}P < 0.001$ compared with histamine after FCCP treatment in (**B**), and between the second histamine treatment and histamine after oligomycin and rotenone in (**D**). The number of experiments is shown in parentheses.

As shown in Figure 2(A), histamine also produced $[Ca^{2+}]_M$ peaks that saturate AEQ1 in an additional 20 % of the mitochondria, but produce a minor response using AEQ2 ($[Ca^{2+}]_M$ approx. 10 μM). The question now arises as to whether these mitochondria also undergo large $[Ca^{2+}]_M$ peaks in response to high K^+ and caffeine or, alternatively, they are located at more internal locations. Figure 6 shows experiments similar to those in Figure 5 but performed with AEQ1. Again, both high K^+ and caffeine abolished the $[Ca^{2+}]_M$ increase and aequorin consumption induced by histamine under these conditions (compare with Figure 2A). This suggests that even these mitochondria responding to histamine with intermediate $[Ca^{2+}]_M$ peaks also appear to be close to Ca^{2+} entry sites or to ryanodine receptors.

Finally, we have studied whether mitochondrial Ca^{2+} uptake modifies histamine-induced catecholamine secretion by investigating the effects of the mitochondrial uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and the combination of mitochondrial inhibitors rotenone and oligomycin on the secretory response induced by histamine. Figure

7(A) shows original traces of amperometric secretory responses elicited by repeated pulsing with 10 μM histamine. It should be observed that the magnitude of such a response decayed considerably from 50 nA in the first pulse to 20 nA in the second one. However, during the third pulse when FCCP (20 μM) was given for 5 s before histamine, the response was enhanced dramatically, reaching a peak of 100 nA. This potentiation is better seen in Figure 7(B), where the means of eight experiments, expressed as the total secretion in microcoulombs, are shown (see Figure legend). The second pulse of histamine evoked 0.3 μC of catecholamine release, whereas the third pulse with FCCP increased this response to as much as 4 μC, more than 10-fold higher.

The same picture emerged when using oligomycin (3 μM) and rotenone (4 μM) to block the uptake of Ca^{2+} into mitochondria, although the potentiation of secretion was lower than with FCCP. Figure 7(C) shows the secretory spikes obtained in a representative experiment, with the mean data from 19 experiments shown in Figure 7(D). In the absence of oligomycin and rotenone, histamine caused a secretory response of 0.4 μC, whereas, after treatment with oligomycin and rotenone, secretion more than doubled to 0.9 μC.

The effects of FCCP and oligomycin and rotenone on histamine-induced $[Ca^{2+}]_c$ peaks were also tested in single-cell $[Ca^{2+}]_c$ imaging experiments. Pretreatment with 20 μM FCCP for 10 s or with oligomycin (3 μM) and rotenone (4 μM) for 90 s produced no significant difference in the height or the kinetics of the $[Ca^{2+}]_c$ peaks. In particular, the rates of return to resting $[Ca^{2+}]_c$ levels were not affected (results not shown).

DISCUSSION

In recent years, results from different laboratories have provided evidence that mitochondria play an important role in intracellular Ca^{2+} homoeostasis. They accumulate large amounts of Ca^{2+} during cell stimulation and constitute probably the most important intracellular buffer of cytosolic Ca^{2+} transients [13,31]. Moreover, mitochondria have also been shown to control the Ca^{2+} inactivation properties of capacitative Ca^{2+} channels [32,33] and voltage-dependent Ca^{2+} channels [34], probably due to its Ca^{2+} buffering capacity. In chromaffin cells, measuring $[Ca^{2+}]_M$ with low-Ca^{2+}-affinity-targeted aequorin, we have shown recently that a subpopulation of mitochondria reached a $[Ca^{2+}]_M$ of approx. 500 μM during stimulation with high K^+ or caffeine [7]. Abolition of this $[Ca^{2+}]_M$ increase with protonophores or mitochondrial toxins dramatically enhanced catecholamine secretion [7,18], suggesting that mitochondria may be a key regulator of neurosecretion. Our experiments with targeted aequorin also suggested the presence in chromaffin cells of complex functional units composed of plasma-membrane voltage-dependent Ca^{2+} channels, ER ryanodine receptors and mitochondria [7]. These functional units would most likely be coupled to secretory vesicles ready to be released, thus controlling the rate of secretion.

In the present study, we have studied the effect of Ca^{2+} release from the ER via $InsP_3$ receptors on mitochondrial Ca^{2+} uptake and catecholamine secretion. Our data suggest that activation of $InsP_3$ receptors produces a highly heterogeneous increase in $[Ca^{2+}]_M$. Subcellular heterogeneity was explored by use of aequorins with different Ca^{2+} affinities and by taking advantage of its large dynamic range and its irreversible consumption during the experiments. These properties allow the $[Ca^{2+}]$ both in regions with the highest $[Ca^{2+}]$ (whose luminescence is so intense than the luminescence from other regions becomes negligible when measured using low-Ca^{2+}-affinity aequorin) and the lowest $[Ca^{2+}]$ (after full consumption of the high-Ca^{2+}-affinity aequorin present in the other compartments) to be measured precisely. This kind of analysis allowed us to distinguish two mitochondrial compartments, one containing approx. 70 % of mitochondria, which respond to histamine with increases in $[Ca^{2+}]_M$ to approx. 1 μM, and another that includes approx. 2–3 % mitochondria, which respond to histamine with large $[Ca^{2+}]_M$ peaks of several hundred micromolar. The remaining mitochondria (approx. 25 %) responded with peaks of intermediate and variable magnitude and, in most cases, were no larger than 10 μM. This heterogeneity was generated by a mixture of cell-to-cell differential $[Ca^{2+}]_M$ response to histamine and subcellular heterogeneity in mitochondrial response. The heterogeneity in $[Ca^{2+}]_M$ response among different cells was larger than that of the $[Ca^{2+}]_c$ response, perhaps because $[Ca^{2+}]_M$ amplifies the $[Ca^{2+}]_c$ changes. Because of this multiple intercellular and subcellular heterogeneity, the different compartments mentioned above may not be identical for each cell. Rather, it is possible that those cells producing a larger $[Ca^{2+}]_M$ response may have a larger proportion of the high-$[Ca^{2+}]_M$ compartments. Given the small percentage of mitochondria that have $[Ca^{2+}]_M$ increases in the hundreds of micromolar range, this kind of strong response to histamine could be present in only some of the cells. However, subcellular heterogeneity persists even in those cells producing a larger response with at least 50 % of their mitochondria responding to histamine with relatively small $[Ca^{2+}]_M$ peaks (approx. 1 μM). Similar findings were obtained when the effect of high K^+ stimulation on $[Ca^{2+}]_M$ was studied at the single-cell level [27].

A relevant question here regards whether the 2–3 % of mitochondrial space that produces the very large $[Ca^{2+}]_M$ peaks corresponds to separate mitochondria, or if it may just reflect spatially defined differences in the Ca^{2+} response of an extended mitochondrial reticulum. If the second alternative were correct, we would expect that diffusion of aequorin along the mitochondrial lumen should be able to restore the luminescence response after the local consumption induced by previous stimulation. This phenomenon was never observed even using times of 10–15 min, and so we conclude that either the mitochondrial reticulum is not completely continuous or, alternatively, that protein diffusion along the lumen is highly restricted.

Another important question was whether or not the mitochondria that responded with high $[Ca^{2+}]_M$ peaks to histamine were the same as those that responded to high K^+ or caffeine stimulation [7]. The results from Figures 4 and 5 show that all the mitochondria able to respond to histamine with $[Ca^{2+}]_M$ peaks larger than 1 μM were also able to produce large $[Ca^{2+}]_M$ responses after stimulation with high K^+ or caffeine. This suggests that $InsP_3$ receptors are also present in the above-mentioned functional units. However, it must be noted that the global $[Ca^{2+}]_M$ increase induced by histamine is much smaller than that produced by high K^+ or caffeine. Caffeine and high K^+ produce $[Ca^{2+}]_M$ peaks in the hundreds of micromolar range in 30–50 % of the mitochondria, whereas similar $[Ca^{2+}]_M$ peaks are only observed in 2–3 % of them after histamine stimulation. Therefore our results suggest that the 30 % of mitochondria that produce $[Ca^{2+}]_M$ peaks above 1 μM after histamine stimulation are the same that respond with near-millimolar $[Ca^{2+}]_M$ peaks to high K^+ or caffeine, but the size of the $[Ca^{2+}]_M$ response induced by histamine is obviously much smaller. Because of the strong $[Ca^{2+}]_c$ and $[Ca^{2+}]_M$ increase induced by high K^+ or caffeine, it could be argued that these stimuli may affect the mitochondrial pool sensitive to histamine even in the absence of functional colocalization. However, this effect would hardly explain the nearly complete abolition of the response to histamine after caffeine or high-K^+ stimulation, unless additional assumptions are made,

e.g. heterogeneous response of individual mitochondria to a similar increase in $[Ca^{2+}]_c$. This is not the case in permeabilized cells, where the whole mitochondrial population responds relatively homogeneously to perfusion of controlled $[Ca^{2+}]$ [7], but this possibility cannot be excluded yet in intact cells. Of course, our data provide only functional evidence for the co-localization of mitochondria, InsP_3 receptors, ryanodine receptors and plasma-membrane Ca^{2+} channels. Definitive demonstration will require morphological studies using immunoelectron microscopy. We should remark that our present (this study) and previous [7] data do not exclude that InsP_3 receptors or ryanodine receptors may also be found widely distributed throughout the ER. Our $[Ca^{2+}]_M$ measurements indicate that these receptors are tightly coupled to mitochondria only in precise locations near the plasmalemma (the functional units). Internal regions of the ER may also have InsP_3 receptors or ryanodine receptors, but they are apparently not tightly coupled to mitochondria.

The presence of InsP_3 receptors in the functional units suggests that they may contribute to the release of Ca^{2+} close to secretory sites. The data in Figure 7 show that abolition of mitochondrial Ca^{2+} uptake with FCCP or mitochondrial inhibitors enhanced the secretory response to histamine. However, the use of protonophores or mitochondrial inhibitors to block mitochondrial Ca^{2+} uptake has the drawbacks of the possible reduction of cellular ATP levels and the possible side-effects of FCCP acting as a protonophore in other cellular targets. To minimize these problems, FCCP was added for only a very short time (5 s) before histamine, and oligomycin was applied together with rotenone to avoid mitochondrial ATPase reversal. The kinetics of the histamine-induced $[Ca^{2+}]_c$ peaks obtained after these treatments were similar to that of the controls, suggesting that the possible variations of ATP levels were not enough to modify the global Ca^{2+} homoeostatic processes. We cannot exclude, however, the presence of variations in ATP levels, perhaps even in local subcellular environments, which could affect secretion but not Ca^{2+} homoeostasis. On the other hand, the effect of FCCP enhancing secretion was much stronger than that of rotenone and oligomycin. This could be due to the faster and stronger mitochondrial depolarization induced by this agent compared with the mitochondrial inhibitors, but could also be attributed to additional non-specific effects of the protonophore. We should therefore be cautious with regard to the interpretation of these experiments. Unfortunately, at the present moment, there are no alternative means to inhibit mitochondrial Ca^{2+} uptake in intact cells. The lack of effect of FCCP or the mitochondrial inhibitors on the histamine-induced $[Ca^{2+}]_c$ peaks contrasts with the increase observed in the high-K^+-induced $[Ca^{2+}]_c$ peaks in the presence of protonophores [35]. The reason is probably that the amount of Ca^{2+} sequestered by mitochondria is much smaller after stimulation with histamine, so that global $[Ca^{2+}]_c$ changes are not significantly affected.

In conclusion, our present and previous data draw a novel view regarding the role of organelles in neurosecretion. The ER is a large Ca^{2+} store able to potentiate secretion by releasing Ca^{2+} near the secretory sites by either Ca^{2+}-induced Ca^{2+} release [30] or activation of InsP_3 receptors (the present study). On the other hand, mitochondria strategically located close to these sites act as a damper of the local Ca^{2+} transients and consequently reduce the magnitude of the secretory response [7]. In contrast, Ca^{2+} uptake by mitochondria also serves to protect plasma-membrane Ca^{2+} channels from Ca^{2+}-dependent inactivation [34]. This mechanism should help to keep the channels open, increase the duration of the Ca^{2+} entry and enhance the secretory response. The interplay between these effects of the ER and mitochondria probably constitutes a key factor in the modulation of secretion and perhaps in the generation of synaptic plasticity. Moreover, alterations in these complex organelle inter-relationships may also underlie the development of a variety of neurodegenerative diseases.

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REFERENCES

1 Rizzuto, R., Pinton, P., Carrington, W., Fay, F. S., Fogarty, K. E., Lifshitz, L. M., Tuft, R. A. and Pozzan, T. (1998) Close contacts with the endoplasmic reticulum as determinants of mitochondrial Ca^{2+} responses. Science (Washington, D.C.) **280**, 1763–1766
2 Mannella, C. A., Buttle, K., Rath, B. K. and Marko, M. (1998) Electron microscopic tomography of rat-liver mitochondria and their interaction with the endoplasmic reticulum. BioFactors **8**, 225–228
3 Frey, T. G. and Mannella, C. A. (2000) The internal structure of mitochondria. Trends Biochem. Sci. **25**, 319–324
4 Hajnoczky, G., Csordas, G., Madesh, M. and Pacher, P. (2000) The machinery of local Ca^{2+} signalling between sarco-endoplasmic reticulum and mitochondria. J. Physiol. (Cambridge, U.K.) **529**, 69–81
5 Park, M. K., Ashby, M. C., Erdemli, G., Petersen, O. H. and Tepikin, A. V. (2001) Perinuclear, perigranular and sub-plasmalemmal mitochondria have distinct functions in the regulation of cellular calcium transport. EMBO J. **20**, 1863–1874
6 Pivovarova, N. B., Hongpaisan, J., Andrews, S. B. and Friel, D. D. (1999) Depolarization-induced mitochondrial Ca accumulation in sympathetic neurons: spatial and temporal characteristics. J. Neurosci. **19**, 6372–6384
7 Montero, M., Alonso, M. T., Carnicero, E., Cuchillo-Ibanez, I., Albillos, A., Garcia, A. G., Garcia-Sancho, J. and Alvarez, J. (2000) Chromaffin-cell stimulation triggers fast millimolar mitochondrial Ca^{2+} transients that modulate secretion. Nat. Cell Biol. **2**, 57–61
8 Rizzuto, R., Brini, M., Murgia, M. and Pozzan, T. (1993) Microdomains with high Ca^{2+} close to IP_3-sensitive channels that are sensed by neighboring mitochondria. Science (Washington, D.C.) **262**, 744–747
9 Rizzuto, R., Bastianutto, C., Brini, M., Murgia, M. and Pozzan, T. (1994) Mitochondrial Ca^{2+} homeostasis in intact cells. J. Cell Biol. **126**, 1183–1194
10 Csordas, G., Thomas, A. P. and Hajnoczky, G. (1999) Quasi-synaptic calcium signal transmission between endoplasmic reticulum and mitochondria. EMBO J. **18**, 96–108
11 Werth, J. L. and Thayer, S. A. (1994) Mitochondria buffer physiological calcium loads in cultured rat dorsal root ganglion neurons. J. Neurosci. **14**, 348–356
12 Park, Y. B., Herrington, J., Babcock, D. F. and Hille, B. (1996) Ca^{2+} clearance mechanisms in isolated rat adrenal chromaffin cells. J. Physiol. (Cambridge, U.K.) **492**, 329–346
13 Herrington, J., Park, Y. B., Babcock, D. F. and Hille, B. (1996) Dominant role of mitochondria in clearance of large Ca^{2+} loads from rat adrenal chromaffin cells. Neuron **16**, 219–228
14 Babcock, D. F., Herrington, J., Goodwin, P. C., Park, Y. B. and Hille, B. (1997) Mitochondrial participation in the intracellular Ca^{2+} network. J. Cell Biol. **136**, 833–844
15 White, R. J. and Reynolds, I. J. (1997) Mitochondria accumulate Ca^{2+} following intense glutamate stimulation of cultured rat forebrain neurones. J. Physiol. (Cambridge, U.K.) **498**, 31–47
16 Brini, M., De Giorgi, F., Murgia, M., Marsault, R., Massimino, M. L., Cantini, M., Rizzuto, R. and Pozzan, T. (1997) Subcellular analysis of Ca^{2+} homeostasis in primary cultures of skeletal muscle myotubes. Mol. Biol. Cell **8**, 129–143
17 Rizzuto, R., Bernardi, P. and Pozzan, T. (2000) Mitochondria as all-round players of the calcium game. J. Physiol. (Cambridge, U.K.) **529**, 37–47
18 Giovannucci, D. R., Hlubek, M. D. and Stuenkel, E. L. (1999) Mitochondria regulate the Ca^{2+}-exocytosis relationship of bovine adrenal chromaffin cells. J. Neurosci. **19**, 9261–9270
19 Moro, M. A., Lopez, M. G., Gandia, L., Michelena, P. and Garcia, A. G. (1990) Separation and culture of living adrenaline- and noradrenaline-containing cells from bovine adrenal medullae. Anal. Biochem. **185**, 243–248
20 Rizzuto, R., Simpson, A. W., Brini, M. and Pozzan, T. (1992) Rapid changes of mitochondrial Ca^{2+} revealed by specifically targeted recombinant aequorin. Nature (London) **358**, 325–327
21 Brini, M., Marsault, R., Bastianutto, C., Alvarez, J., Pozzan, T. and Rizzuto, R. (1995) Transfected aequorin in the measurement of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$). A critical evaluation. J. Biol. Chem. **270**, 9896–9903

22 Montero, M., Brini, M., Marsault, R., Alvarez, J., Sitia, R., Pozzan, T. and Rizzuto, R. (1995) Monitoring dynamic changes in free Ca^{2+} concentration in the endoplasmic reticulum of intact cells. EMBO J. **14**, 5467–5475
23 Alonso, M. T., Barrero, M. J., Carnicero, E., Montero, M., Garcia-Sancho, J. and Alvarez, J. (1998) Functional measurements of $[Ca^{2+}]$ in the endoplasmic reticulum using a herpes virus to deliver targeted aequorin. Cell Calcium **24**, 87–96
24 Montero, M., Barrero, M. J. and Alvarez, J. (1997) $[Ca^{2+}]$ microdomains control agonist-induced Ca^{2+} release in intact HeLa cells. FASEB J. **11**, 881–885
25 Barrero, M. J., Montero, M. and Alvarez, J. (1997) Dynamics of $[Ca^{2+}]$ in the endoplasmic reticulum and cytoplasm of intact HeLa cells. A comparative study. J. Biol. Chem. **272**, 27694–27699
26 Villalobos, C., Nuñez, L., Chamero, P., Alonso, M. T. and García-Sancho, J. (2001) Mitochondrial $[Ca^{2+}]$ oscillations driven by local high-$[Ca^{2+}]$ domains generated by spontaneous electric activity. J. Biol. Chem. **276**, 40293–40297
27 Villalobos, C., Nuñez, L., Montero, M., García, A. G., Alonso, M. T., Chamero, P., Alvarez, J. and García-Sancho, J. (2002) Redistribution of Ca^{2+} among cytosol and organella during stimulation of bovine chromaffin cells. FASEB J. **16**, 343–353
28 Nuñez, L., De la Fuente, M. T., Garcia, A. G. and Garcia-Sancho, J. (1995) Differential Ca^{2+} responses of adrenergic and noradrenergic chromaffin cells to various secretagoges. Am. J. Physiol. **269**, C1540–C1546
29 Borges, R., Sala, F. and Garcia, A. G. (1986) Continuous monitoring of catecholamine release from perfused cat adrenals. J. Neurosci. Methods **16**, 289–300
30 Alonso, M. T., Barrero, M. J., Michelena, P., Carnicero, E., Cuchillo, I., Garcia, A. G., Garcia-Sancho, J., Montero, M. and Alvarez, J. (1999) Ca^{2+}-induced Ca^{2+} release in chromaffin cells seen from inside the ER with targeted aequorin. J. Cell Biol. **144**, 241–254
31 Xu, T., Naraghi, M., Kang, H. and Neher, E. (1997) Kinetic studies of Ca^{2+} binding and Ca^{2+} clearance in the cytosol of adrenal chromaffin cells. Biophys. J. **73**, 532–545
32 Hoth, M., Button, D. C. and Lewis, R. S. (2000) 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**, 10607–10612
33 Wasniewska, M. and Duszynski, J. (2000) The role of mitochondrial dysfunction in regulation of store-operated calcium channels in glioma C6 and human fibroblast cells. FEBS Lett. **478**, 237–240
34 Hernandez-Guijo, J. M., Maneu-Flores, V. E., Ruiz-Nuno, A., Villarroya, M., Garcia, A. G. and Gandia, L. (2001) Calcium-dependent inhibition of L, N, and P/Q Ca^{2+} channels in chromaffin cells: role of mitochondria. J. Neurosci. **21**, 2553–2560
35 Montero, M., Alonso, M. T., Albillos, A., García-Sancho, J. and Alvarez, J. (2001) Mitochondrial Ca^{2+}-induced Ca^{2+} release mediated by the Ca^{2+} uniporter. Mol. Biol. Cell **12**, 63–71

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