Stable Interactions between Mitochondria and Endoplasmic Reticulum Allow Rapid Accumulation of Calcium in a Subpopulation of Mitochondria*

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To better understand the functional role of the mitochondrial network in shaping the Ca\(^{2+}\) signals in living cells, we took advantage both of the newest genetically engineered green fluorescent protein-based Ca\(^{2+}\) sensors (“Cameleons,” “Camgaroos,” and “Pericams”) and of the classical Ca\(^{2+}\)-sensitive photoprotein aequorin, all targeted to the mitochondrial matrix. The properties of the green fluorescent protein-based probes in terms of subcellular localization, photosensitivity, and Ca\(^{2+}\) affinity have been analyzed in detail. It is concluded that the ratiometric pericam is, at present, the most reliable mitochondrial Ca\(^{2+}\) probe for single cell studies, although this probe too is not devoid of problems. The results obtained with ratiometric pericam in single cells, combined with those obtained at the population level with aequorin, provide strong evidence demonstrating that the close vicinity of mitochondria to the Ca\(^{2+}\) release channels (and thus responsible for the fast uptake of Ca\(^{2+}\) by mitochondria upon receptor activation) are highly stable in time, suggesting the existence of specific interactions between mitochondria and the endoplasmic reticulum.

Calcium is arguably the most versatile player within the cell. This second messenger is directly or indirectly involved in a myriad of processes that span virtually all physiological aspects of a cell, including its birth, health, disease, and death. From a generic point of view, calcium was often seen to exert its action exclusively through changes in cytosolic free calcium concentration ([Ca\(^{2+}\)]\(_{c}\))\(^{1}\). In recent years, these global changes have been dissected into regional variations, and different organelles have seen their importance accrued. In particular, in practically all cell types investigated, it has been found that the speed and amplitude of mitochondrial Ca\(^{2+}\) uptake depend not only on the amplitude of the [Ca\(^{2+}\)]\(_{c}\) rise, but also on the source of Ca\(^{2+}\) and the mechanisms through which the Ca\(^{2+}\) increase is elicited. Specifically, fast increase of mitochondrial Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{m}\)) can be triggered by either Ca\(^{2+}\) mobilization from stores or Ca\(^{2+}\) influx from the medium, or both, depending on the cell type. Because mitochondrial Ca\(^{2+}\) uptake takes place exclusively through the so-called calcium uniporter, and because this uniporter has a low affinity for Ca\(^{2+}\), it was not clear how the relatively low, average [Ca\(^{2+}\)]\(_{c}\), increase elicited by physiological stimuli could efficiently drive the observed rise in [Ca\(^{2+}\)]\(_{m}\). The concept of high Ca\(^{2+}\) microdomains was thus developed to explain the very rapid Ca\(^{2+}\) uptake by mitochondria under physiological conditions. The hypothesis predicts that the fast [Ca\(^{2+}\)]\(_{m}\) increases depend on the close vicinity of these organelles to Ca\(^{2+}\) channels, where the Ca\(^{2+}\) concentration is sufficiently high to drive an efficient uptake by the low affinity mitochondrial uniporter (1, 2). It is worth noting that this microdomain model does not purport to explain all mitochondrial Ca\(^{2+}\) uptake; rather, the model provides a conceptual framework to explain the fast [Ca\(^{2+}\)]\(_{m}\) rises, erstwhile paradoxical.

Given the motility of both ER and mitochondria, and the requirement for regions of close proximity between the two organelles to ensure a highly efficient mitochondrial Ca\(^{2+}\) uptake, it becomes of paramount importance to understand how this spatial arrangement is obtained. From a structural point of view, the ER is a highly convoluted reticular network, while mitochondria have historically been erroneously depicted as “fuse-like” individual organelles that pepper the cytoplasm. The notion that also mitochondria form a network within the cytoplasm is practically half a century old, but only recently has it been more widely accredited, with in vivo studies that have revealed a highly dynamic network that continuously undergoes multiple fusion and fission processes (2–5). Essentially, two major scenarios can be envisaged. On one hand, the vicinity of mitochondria to Ca\(^{2+}\) release sites may be a stochastic event, because of the abundance and motility of both organelles. On the other, transient or permanent interactions may exist to keep specific mitochondrial subpopulations close to sites where Ca\(^{2+}\) reaches high concentrations. It has been suggested that stable mitochondria-ER interactions might occur in adrenal medullary cells (6), but this phenomenon has not been analyzed in detail. In the present study, we adopted a bipartite approach to explore this issue. For single cell studies we employed the new GFP-based Ca\(^{2+}\) probes selectively targeted to the mitochondrial matrix, whereas at the cell popul-
lution level we took advantage of specific characteristics of aequorin, a Ca\(^{2+}\) probe with an established track record.

**EXPERIMENTAL PROCEDURES**

**Generation of Constructs**—cDNA encoding the N-terminal part (comprising the first 36 amino acids) of subunit VIII of human cytochrome c oxidase was fused, in-frame, to cDNA encoding DsRed (Clontech) to generate mt-DsRed. Mitochondrially targeted versions of Cameleon and split Cameleons were generated similarly. Cameleon, split Cameleons, and mt-Camego-2, and mt- and nPericamR were generous gifts from Roger Y. Tsien and Atsushi Miyawaki, respectively. Details of all constructs are available upon request.

**Cell Cultures and Transfection**—HeLa cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, supplemented with L-glutamine (2 mM), penicillin (100 units/ml), and streptomycin (100 \(\mu\)g/ml), in a humidified atmosphere containing 5% CO\(_2\). Cells transiently expressing aequorin were treated with 20 mM Hepes, pH 7.0, at 37 °C, and supplemented only with digitonin (20 \(\mu\)M); in addition, *Staphylococcus aureus* α-toxin was used (Sigma; 100 \(\mu\)g/ml); cells were maintained in mKRB for the remaining experimental procedures.

Perfusion with histamine (100 \(\mu\)M) was employed to trigger Ca\(^{2+}\) release from intracellular stores. Perfusion with cyclopiazonic acid (20 \(\mu\)M) was employed to inhibit ER Ca\(^{2+}\)-ATPases.

For cytoskeleton disruption, cells were first challenged with histamine and then incubated in KRB, in the presence of colchicine (ICN, Milan, Italy; 10 \(\mu\)g/ml) or cytochalasin D (ICN; 10 \(\mu\)M), or both, for 30 min. The second histamine stimulus was applied in the presence of the same drug(s).

All experimental procedures and incubations were carried out at 37 °C. Chemicals were of analytical or highest available grade and, unless otherwise stated, were acquired from Sigma. Data shown represent typical results obtained in at least five independent experiments; numerical data are presented as mean ± S.D.; statistical significance was calculated by Student’s \(t\) test.

**RESULTS**

**Choice of a Mitochondrial Ca\(^{2+}\) Indicator for Single Cell Studies**—The positively charged fluorescent indicator rhod-2 is often considered the indicator of choice to measure changes in mitochondrial [Ca\(^{2+}\)] at the single cell level. Although this probe has been extensively used by different groups, its subcellular localization is far from ideal; most likely because of this, its use has given rise to partially contradictory results in different cell types (7–10). The search for alternative calcium indicators has prompted the development of a number of genetically encoded fluorescent probes. These probes, however, have yet to be thoroughly characterized. Below we consider the three families of fluorescent indicators that have been recently introduced, i.e. Cameleons, Camgaroos, and Pericams.

Cameleon is a GFP-based Ca\(^{2+}\) indicator, which was initially introduced as a Ca\(^{2+}\) probe for Ca\(^{2+}\) in the cytoplasm, ER, and nucleus (11). A mitochondrial cameleon has been generated by fusing at the N terminus of the cytosolic construct the targeting sequence of subunit VIII of human cytochrome c oxidase (12). This particular mitochondrial targeting presequence has been repeatedly shown to deliver fused proteins in an highly efficient and selective manner to the mitochondrial matrix (13, 14). In a series of elegant experiments (12), it was observed that a cameleon targeted to mitochondria by this strategy only localizes efficiently in cells that express very low levels of the construct. We also confirmed that this fusion protein fails to localize correctly and transfected cells exhibit fluorescence throughout the cytoplasm; only rarely was a selective labeling of mitochondria observed.

An attempt to bypass this problem was to use the so-called split cameleon (11), in which two functional constituents of the cameleon are expressed individually. The two halves of the indicator were targeted to the mitochondrial matrix independently, using the same presequence as before. In this case, the M13-YFP moiety was efficiently delivered exclusively to the cytoplasm; only rarely was a selective labeling of mitochondria observed.

Camgaroo is an insertional mutant of GFP, sensitive to Ca\(^{2+}\) (15). A mitochondrial version of this probe (mtCamgaroo-2; Ref. 16) possesses an N-terminal presequence that effectively targets it to the mitochondrial matrix (Fig. 1A, left panel); targeting of the CFP-CaM part was again deficient, although, with −50% of the fluorescence present throughout the cell body (Fig. 1A, right panel). The reasons for this behavior are not clear at present. Because of these difficulties, no further attempt to characterize mtCameleons was undertaken.

Camgaroo is an insertional mutant of GFP, sensitive to Ca\(^{2+}\) (15). A mitochondrial version of this probe (mtCamgaroo-2; Ref. 16) possesses an N-terminal presequence that effectively targets it to the mitochondrial matrix (Fig. 1B), although in strongly fluorescent cells a residual signal is often present throughout the cell cytoplasm. Results obtained by Tsien and co-workers (16) indicated that mtCameleons-2 can be used to monitor changes in [Ca\(^{2+}\)]\(_{i}\). Surprisingly, in our hands, HeLa cells expressing mtCameleons-2 transiently showed marginal,
often undetected, changes in fluorescence when challenged with histamine (see below). The reason for this discrepancy lies in the illumination protocol used. When cells were illuminated continuously, a very rapid drop (to ~50% of the initial F) was observed; this drop was not due to photobleaching because a short recovery (30 s) in the dark resulted in almost complete recovery of the initial F (Fig. 2A, upper panel). Importantly, the photoconverted form of the probe was almost completely insensitive to Ca^{2+} changes, as indicated by the lack of increase in F upon histamine addition after ~10 s of continuous illumination (Fig. 2A, upper panel). If, on the contrary, the time of illumination was reduced to a minimum and at least 1 s between two successive illuminations were allowed, a clear increase in F was revealed by mtCamgaroo-2 upon histamine challenge (Fig. 2A, lower panel), on average about 20% of the initial value. Under the same conditions, a non-Ca^{2+}-sensitive, but pH-sensitive, YFP gave no change in signal (Fig. 2A, lower panel).

Using mtCamgaroo-2, we observed a substantial subcellular heterogeneity in peak Ca^{2+} increases in single HeLa cells challenged with histamine (Fig. 2B), in agreement with previous studies with rhod-2 or mtCameleons (7, 12). With probes such as rhod-2 or mtCamgaroo-2 (that respond to alterations in [Ca^{2+}]_{i}) simply by changing F, changes in size and shape of organelles, as well as organelle movement, may all create artifacts that confound both quantitative and qualitative analyses (17).

To overcome these limitations, we shifted our attention to the most recent genetically targeted Ca^{2+} probe family (the Pericams; Ref. 18). Fig. 1C shows the typical staining pattern of a HeLa cell transiently expressing a mitochondrionally targeted ratiometric pericam (mtPericamR). The staining is highly specific and completely overlaps with that of a mitochondrially targeted red variant of GFP (mtDsRed). In addition, the nuF is almost indistinguishable from the background of non-transfected cells.

The use of mtPericamR greatly diminishes the problem of photoconversion that plagues mtCamgaroo-2. Upon almost continuous illumination there is still an initial, rapid decrease in F at both wavelengths (but only ~15%); as in the case of mtCamgaroo-2, the initial F is recovered after brief periods of non-illumination (data not shown). The significant difference in relation to mtCamgaroo-2 is that this probe retains its Ca^{2+} sensitivity even during periods of rapid data acquisition (see below).
Characterization of mtPericamR—HeLa cells expressing mtPericamR and challenged with histamine show a subcellular heterogeneous response, as reported previously using other non-ratiometric indicators, confirming that the differences between mitochondrial populations are real and not artifacts of movement. Fig. 3A shows pseudocolor-rendered ratios of a HeLa cell before and after a histamine challenge. When different subsets of mitochondria are selected for analyses, a clear heterogeneity is observed (Fig. 3B).

We next addressed the problem of the relationship between mitochondrial and cytoplasmic \( [Ca^{2+}] \) changes in the same cell. Given that it has been repeatedly demonstrated in HeLa cells that the nucleus behaves essentially as the cytoplasm, we cotransfected these cells with a variant of PericamR targeted to the nucleus (nuPericamR) and mtPericamR. Under resting conditions, the ratio value was higher in the latter. This most likely reflects a difference in resting pH, rather than in \( [Ca^{2+}] \) (see below). Upon a histamine challenge both compartments showed similar kinetics: a sharp rise in the \( F \) ratio, followed by a declining plateau, often accompanied by oscillations (Fig. 3C). Quantitatively, it was surprising to note that this increase was larger in the nucleus than in the mitochondria. This result is in sharp contrast with previous data obtained with aequorin, rhod-2, and mtCameleon, with all these probes, the increase in \( [Ca^{2+}] \) has been found to be larger within mitochondria than in the nucleus (and cytoplasm).

To verify whether this contradiction was because of a different affinity of the probe for \( Ca^{2+} \) in the two compartments, we carried out a calibration in situ with the widely used ionomycin and high \( Ca^{2+} \) method (e.g., Ref. 16). As shown in Fig. 4A (upper panel), both compartments exhibit a sharp increase in the ratio upon histamine stimulation, which returns to basal levels after the agonist is washed away; addition of \( Ca^{2+} \) in the presence of the ionophore also causes an increase in the ratio, this time clearly biphasic: a first rapid increase, followed by a larger and slower rise. However, analysis of the two individual wavelengths shows distinct pictures in the case of histamine and ionomycin. In the former, the increase in ratio is because of a small increase in \( F \) upon excitation at 490 nm and a larger decrease in \( F \) upon excitation at 415 nm (Fig. 4A, lower panel). In the latter, the first rise is similar to that observed for histamine; the second rise, however, is fully because of an increase in \( F \) upon excitation at 490 nm, whereas the 415-nm signal remains essentially unchanged. Given that alkalization increases the \( F \) of PericamR at 490 nm (18), the possibility of a pH artifact in the effect of ionomycin was thus considered. Cells expressing nuPericamR and mtPericamR were challenged with NH4Cl to increase the pH inside the cells without...
It was more difficult to perform the same calibration procedure in the nucleus, because the digitonin treatment (100 μM, as used for calibrating mtPericamR) generally provoked the release of the nuclear probe. We tried to permeabilize the cells with *S. aureus* α-toxin (19) but the results were unsatisfactory, given that very high concentrations of the toxin (~100 μg/ml) resulted only in partial release of a trapped fluorescence probe such as fura-2. However, at lower digitonin concentrations (20 μM), complete release of fura-2 was obtained, whereas in the majority of the cells a large part of nuPericamR remained localized in the nucleus. Accordingly, the \( K'_{\text{S}} \) of nuPericamR in situ was calculated to be ~2.5 μM, similar to that calculated in vitro.

The kinetics of the mitochondrial Ca\(^{2+}\) increase with respect to that in the nucleus were analyzed in the experiments presented in Fig. 6. The high rate of data acquisition permitted by the use of PericamR enabled us to acquire data points (F ratio upon excitation at two independent wavelengths) every 180–200 ms. Detailed analyses of the ratio increases immediately following histamine stimulation revealed different types of heterogeneity. In some cells, the mitochondrial response time was homogeneous, but with different rates of Ca\(^{2+}\) accumulation (Fig. 6A). In others, mitochondria have similar rates of Ca\(^{2+}\) uptake, but different delays in relation to the nuclear Ca\(^{2+}\) rise (data not shown). In general, the heterogeneity observed among different mitochondrial populations, in terms of the lag time between nucleoplasmic and mitochondrial Ca\(^{2+}\) increases, ranged from not appreciable (less than 200 ms) to well above 400 ms. On the other hand, in accordance with previously published results (18), the peak response of the mitochondria was reached with some delay (3–5 s) with respect to that in the nucleus (1–2 s).

**Stochastic or Regulated ER-Mitochondria Interactions?**—Experiments presented above and previously published data confirm a substantial heterogeneity in the mitochondrial response to the Ca\(^{2+}\) increases elicited by an agent such as histamine (that by producing Ins\(_P_3\) mobilizes Ca\(^{2+}\) from intracellular stores). The accepted interpretation for such heterogeneity (and for the speed and amplitude of mitochondrial Ca\(^{2+}\) increases) is that some of the organelles are very close to the Ins\(_P_3\)-gated channels. In this way, they are exposed not to the mean increases in cytoplasmic [Ca\(^{2+}\)] but rather to the microdomains of much higher [Ca\(^{2+}\)] that are formed close to the channels themselves. The still unanswered, but key question, is whether this vicinity is simply a stochastic event of two organelles that are densely packed within the cell (whereby the random movement and reorganization of both networks ensure that some mitochondria are always close to some Ins\(_P_3\) receptors at any given time), or whether more stable associations occur between the two organelles. To address this question, a protocol of double stimulation with histamine was devised. The rationale is as follows. If the vicinity of mitochondria to the Ins\(_P_3\) receptors is stochastic, one would expect that if two similar Ca\(^{2+}\) releases from stores are triggered, the mitochondria that respond maximally to each release will be different in each case. Conversely, if there is a stable association between the two organelles at specific sites, the same subset of mitochondria will show a maximal response in two successive stimulations.

Preliminary to such an approach is the demonstration that under the chosen experimental conditions there is no desensitization of the overall mitochondrial Ca\(^{2+}\) uptake upon two successive histamine stimulations (7). To analyze this aspect in detail, we transfected cells with both nu- and mtPericamR, and the cells were subjected to a double histamine challenge,
changes of the 490/415 nm ratio in a single typical cell, upon addition of 
the presence of FCCP for the remaining experimental procedures.

A  

![Graph](image)

**Fig. 5.** In situ calibration of mtPericamR. HeLa cells were transfected with mtPericamR (panels A and B) or nuPericamR (panel C). In the case of mtPericamR, the cells were incubated in mKRB, buffered at pH 8, supplemented with 100 μM EGTA; digitonin (100 μM) and FCCP (4 μM) were then added and the cells were maintained in the continuous presence of FCCP for the remaining experimental procedures. A, kinetic changes of the 490/415 nm ratio in a single typical cell, upon addition of increasing concentrations of calcium (the total Ca^{2+} in the perfusion 

medium was measured by flame photometry). B, mean 490/415 nm ratio values as a function of [Ca^{2+}] from 14 cells in five different experiments. The values are expressed as percentage of the maximal 490/415 nm ratio (Ratiomax), obtained in each experiment by addition of 300 μM Ca^{2+}; further increases in Ca^{2+} concentration did not result in additional changes in this ratio. C, mean 490/415 nm ratio values as a function of [Ca^{2+}], using nuPericamR. In this case, no FCCP was used and digitonin was kept at 20 μM (see also “Experimental Procedures”).

The constant mobility of the mitochondrial network rendered the proposed analysis problematic. Indeed, when single organelles eliciting a maximal response were selected at any given time point and compared with subsequent frames, it was impossible to determine whether differences observed were because of modified responses of the organelles, or to their movement. Fig. 6C exemplifies this problem. First, pixels with maximal responses to the first stimulus were selected and color-coded as red (panel 1). The pixels of maximal response to the second stimulus were again selected and now color-coded as green (panel 2). Merging of panels 1 and 2 reveals that the pixels of maximal response that colocalize perfectly (color-coded as yellow) represent only a small fraction of the total. This analysis, however, is biased by the fact that the organelles are in continuous movement and accordingly the same mitochondrion could be in a slightly different position at different moments in time.

If a less detailed analysis was carried out, for example by identifying distinct mitochondrial regions, each comprising 3–5% of the total mitochondrial area, the regions that exhibited larger rises upon the first stimulus were generally those that responded maximally upon the second stimulus (data not shown). This type of analysis, however, appears not accurate enough to clearly distinguish whether there is indeed a stable interaction between specific ER regions and individual mitochondria.

**Subcellular Heterogeneity in Mitochondrial Ca^{2+} as Revealed by Targeted Aequorin**—To further address the problem of stochastic versus specific ER-mitochondria interactions, we took advantage of another selective mitochondrial Ca^{2+} probe, aequorin. Aequorin is best suited to monitor changes in cell populations, but some of its characteristics, often overlooked under routine use, makes it ideal to address the problem of stochastic versus regulated ER-mitochondria interactions. In fact, when in contact with Ca^{2+}, aequorin emits light and in the process is transformed into a Ca^{2+}-insensitive, non-luminescent form; in practical terms, the probe is “consumed” in the presence of Ca^{2+}. We took advantage of this characteristic to address the question of whether associations between mitochondria and the ER are maintained over time. The rationale is as follows. Triggering Ca^{2+} release from the ER exposes a subset of the mitochondrial network to microdomains of high [Ca^{2+}]; this subpopulation will uptake a large amount of Ca^{2+} and aequorin will be selectively and markedly consumed. When a second ER Ca^{2+} release event is triggered, two scenarios can be envisaged. If ER and mitochondria are tightly associated, then the mitochondrial regions that undergo massive uptake of Ca^{2+} will be essentially the same; if, on the other hand, both organelles have free mobility, then random regions of mito-
Mitochondria will uptake this Ca\(^{2+}\). In terms of aequorin response, these two situations will generate radically different data. If the former is true, in the highly responding population of mitochondria the percentage of aequorin molecules capable of releasing photons will be greatly reduced (having been largely consumed during the exposure to the first Ca\(^{2+}\) burst), and the emitted light will be drastically diminished. On the other hand, if the latter is true, essentially the same quantity of aequorin molecules will be available to release photons, and the light signal generated should be comparable with that observed during the first Ca\(^{2+}\) burst. The calibrated Ca\(^{2+}\) signal (that takes into account the average consumption of aequorin) would appear drastically reduced upon the second histamine challenge in the first case and essentially unmodified in the second. As shown in Fig. 7A, the amplitude of the second response is only marginally diminished.

It should be noted that the experiments carried out with

![Figure 6](image-url)

**Fig. 6. Heterogeneity of mitochondrial Ca\(^{2+}\) responses monitored with PericamR.** A, HeLa cells were cotransfected with mtPericamR and nuPericamR, stimulated with histamine (100 \(\mu\)M), and monitored at 415 and 490 nm (for 100 and 50 ms, respectively; a pair of images was acquired every 180 ms). Where indicated, 100 \(\mu\)M histamine was added. Regions of interest were selected, defining parts of the nucleus and mitochondrial network. Fluorescence ratios (490/415 nm) were normalized to the maximal response observed in each region. The expanded graph represents the boxed region of the inset and shows traces of distinct mitochondrial (mt) and nuclear (nu) regions. B, HeLa cells cotransfected with mtPericamR and nuPericamR were subjected to a double histamine challenge at different time points. The value of the second response is shown as a percentage of the first. Values are mean ± S.D.; the number of cells analyzed ranged from 12 to 52, with no more than 3 cells per independent experiment; no significant difference between the first and second challenge was observed at any time point. C, HeLa cells expressing mtPericamR were subjected to two 30-s applications of 100 \(\mu\)M histamine, applied 7 min apart. Pixels of maximal response (top 30% of response range) were selected from the ratio images after the first (1) and second (2) histamine challenges (shown in red and green, respectively). The overlay of the two selections is presented in panel 3.
The standard protocol were challenged twice with histamine (100 nM). Aequorin was expressed in the cytosol (cytAequorin) or mitochondria (mtAequorin) and reconstituted with coelenterazine according to the standard protocol. The expressed apoaequorin becomes fully functional (i.e., capable of emitting light, and being consumed), in the presence of Ca2+ only after being covalently linked to coelenterazine. This latter process is generally referred to as “reconstitution” and is usually carried out over a period of hours, during which cells expressing apoaequorin are bathed in medium containing coelenterazine.

We ascertained that the minimum loading time that still ensures a reliable light signal could be lowered to 5 min and consequently developed the following experimental protocol. HeLa cells expressing wild-type apoaequorin, but without added coelenterazine, were challenged with histamine (no light output was obviously detected under these conditions, and aequorin is not consumed); histamine was washed away and coelenterazine loaded acutely for 5 min. Using this short reconstitution protocol, the mitochondria are exposed to two consecutive fast Ca2+ increases, but the selective consumption of aequorin during the first peak is avoided.

As shown in Fig. 7B, the amplitude of the mitochondrial response because of a second histamine challenge applied 9 min after the first, and measured by the freshly reconstituted aequorin, was almost 80% of that observed during a first challenge. Under the same conditions, the response of cells reconstituted with the standard protocol (i.e., incubation with coelenterazine for two hours prior to stimulation) was about 45% of the first. These data further support the notion that the drastic reduction of the [Ca2+]i increase observed upon a second challenge with histamine is largely because of a selective consumption of the probe and not to the desensitization of the mitochondrial uniporter.

To characterize in more detail the dynamics of aequorin responses both in the cytosol and in the mitochondrial matrix, measurements were made at different time points. As shown in Fig. 7C, when the second histamine pulse was applied 5 min after the second, the response was diminished in both compartments, but more drastically within mitochondria. From here onwards, the two compartments behaved very distinctively. Whereas the cytosolic response recovered to practically normal levels within 10–15 min, the mitochondrial response took much longer to recover. Indeed, even when the second histamine pulse was delivered 90 min after the first, the peak amplitude still showed a 15% reduction.

To test whether the stability of the association between the two organelles depends on cytoskeleton integrity, the cells were treated with cytochalasin D or colchicine or both. The amplitude of the Ca2+ response (30 min after the first challenge) in control experiments (7.49 ± 2.59 μM; n = 8), and upon treatment with cytochalasin D (6.39 ± 1.85 μM; n = 7), colchicine (6.37 ± 1.54 μM; n = 10), or both (7.97 ± 1.26 μM; n = 5), did not reveal significant differences. The data represents mean ± S.D. of nine and eight independent experiments, respectively. C, HeLa cells expressing cytAequorin or mtAequorin were challenged twice with histamine, at varying intervals. The graph displays the intensity of the second peak response (as a percentage of the first), expressed as mean ± S.D.; significant differences between cytosolic and mitochondrial values are indicated by ** (p < 0.01) and * (p > 0.05).

Fig. 7. Double stimulation with histamine of HeLa cells transfected with cytosolic or mitochondrial aequorin. A, HeLa cells expressing aequorin targeted to the cytosol (cytAequorin) or mitochondria (mtAequorin) and reconstituted with coelenterazine according to the standard protocol were challenged twice with histamine (100 μM) in KRB, with an interval of 7 min between the two stimuli. B, HeLa cells expressing mtPericamR (see above) exclude the possibility that the rapid drop in the mitochondrial Ca2+ response is because of a desensitization of the mitochondrial uniporter. To address this issue in more detail, we took advantage of another particularity of the aequorin system. The expressed apoaequorin becomes fully functional (and, thus, capable of emitting light, and being consumed, in the presence of Ca2+ only after being covalently linked to coelenterazine. This latter process is generally referred to as “reconstitution” and is usually carried out over a period of hours, during which cells expressing apoaequorin are bathed in medium containing coelenterazine. We ascertained that the minimum loading time that still ensures a reliable light signal could be lowered to 5 min and consequently developed the following experimental protocol. HeLa cells expressing wild-type apoaequorin, but without added coelenterazine, were challenged with histamine (no light output was obviously detected under these conditions, and aequorin is not consumed); histamine was washed away and coelenterazine loaded acutely for 5 min. Using this short reconstitution protocol, the mitochondria are exposed to two consecutive fast Ca2+ increases, but the selective consumption of aequorin during the first peak is avoided.

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Mitochondria-ER Interactions and Ca\(^{2+}\) Signaling

![Fig. 7.](image)

![Fig. 8.](image)

**Fig. 8.** Effect of Ca\(^{2+}\) mobilization and Ca\(^{2+}\) influx on [Ca\(^{2+}\)]\(_i\).

- **A**
  - EGTA
  - EGTA

- **B**
  - CPA
  - Histamine

HeLa cells transfected with mtAequorin were incubated in KRB. Where indicated, the perfusing medium containing 1 mM CaCl\(_2\) was substituted with Ca\(^{2+}\)-free medium supplemented with 100 μM EGTA. The concentrations of histamine and cyclopiazonic acid (CPA) were 100 and 20 μM, respectively. Other conditions are as described in the legend to Fig. 7.

Taken together, the experiments carried out both at the single cell and at the population level make a strong case in favor of the existence of a stable, highly responsive, subpopulation of mitochondria. It could be argued that this highly responsive mitochondrial subpopulation takes up Ca\(^{2+}\) more efficiently than the rest not because of its vicinity to the Ca\(^{2+}\) release sites, but because its membrane potential (and thus the driving force for Ca\(^{2+}\) accumulation) is higher (20). Were this the case, the same subpopulation of mitochondria would accumulate Ca\(^{2+}\) with high efficiency, irrespective of the mechanism leading to the increase in [Ca\(^{2+}\)]\(_i\). To test this hypothesis, cytosolic Ca\(^{2+}\) was increased in two different ways: by triggering efflux from the ER (via the InsP\(_3\) receptors), and by activating influx across the plasma membrane (through store-operated Ca\(^{2+}\) channels).

The results presented in Fig. 8, panel A, show that the peak amplitude of the mitochondrial Ca\(^{2+}\) uptake, elicited by Ca\(^{2+}\) readdition to cells treated with cyclopiazonic acid (an inhibitor of the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase; the Ca\(^{2+}\) influx is thus through the capacitative pathway), was hardly changed by a pre-challenge with histamine. Similarly, the Ca\(^{2+}\) peak because of mobilization from stores (i.e., elicited by histamine in Ca\(^{2+}\)-free medium) was virtually unaffected by a previous activation of Ca\(^{2+}\) influx (panel B). In a series of experiments, the peak because of capacitative Ca\(^{2+}\) influx following a histamine challenge was ~87% of that elicited without any histamine challenge (3.87 ± 0.45 versus 4.45 ± 0.23 μM; n = 4 and 3, respectively). Similarly, the peak because of Ca\(^{2+}\) mobilization showed a negligible difference to controls when elicited after the activation of capacitative influx (9.62 ± 1.05 versus 9.42 ± 1.55 μM; n = 4 and 3, respectively). For comparison, in the inset of Fig. 8, panel A, the major apparent reduction observed in the same batch of cells in a double histamine challenge is presented. Of interest, a double activation of Ca\(^{2+}\) influx through the capacitative pathway resulted in a substantial apparent reduction (>50%) of the second response; this phenomenon was not reversed even when the second challenge was delayed by as much as 15–20 min (data not shown).

**DISCUSSION**

Since the initial discovery of rapid and efficient Ca\(^{2+}\) uptake by mitochondria in intact cells, numerous fluorescent probes have been introduced to investigate in situ the properties of the uptake and release mechanisms, and the impact of mitochondrial Ca\(^{2+}\) handling on the overall Ca\(^{2+}\) signaling characteristics. The search for a reliable mitochondrial Ca\(^{2+}\) indicator has been particularly intense in the last few years. None of the available probes appear ideal and all have different advantages and disadvantages. The use of rhod-2 is straightforward and the molecule can be loaded into virtually any cell. However, this dye is plagued by two major problems: it is partially mistargeted (depending on the cell type and the loading protocol), and responds to Ca\(^{2+}\) changes simply with changes in F (i.e., it is not ratiometric). An additional problem that has been rarely (or not at all) addressed is the Ca\(^{2+}\) buffering capacity of rhod-2. The possible influence of the dye in reducing the amplitude of [Ca\(^{2+}\)]\(_i\) increases should be carefully considered.

Molecularly engineered GFP-based fluorescent Ca\(^{2+}\) probes (Cameleons, Camgaroos, and Pericams), on the other hand, are theoretically superior to rhod-2 in terms of selectivity of localization and Ca\(^{2+}\) buffering capacity. But they are not trouble-free either: transfection is required (some cell types could therefore be more difficult to use), and the functional characterization of these probes as mitochondrial Ca\(^{2+}\) indicators is still in its primordial stages.

We observed that mtCameleon has unexpected major problems of localization. Only in cells with very low levels of expression (and not always even in these) a selective targeting to the mitochondria was observed. We feel that this limitation is so important that we decided not to characterize this probe further.

Unlike mtCameleon, mtCamgaroo-2 is well directed to the matrix, although not as efficiently as mtGFP or mtAequorin. In fact, whereas the levels of mtGFP or mtAequorin in the nucleus and cytosol are below detection level, in the case of mtCamgaroo-2 the nuclear signal is 5–20% of that measured in mitochondria. The reasons for the partial mistargeting of calmodulin-containing GFP fusion proteins is presently unknown. Yet partial mistargeting is not the major problem of mtCamgaroo-2 as a Ca\(^{2+}\) indicator, because the photoconversion phenomenon is by far more troublesome. Photoconversion is a known property of wild type GFP (21), but it has not been reported for GFP mutants. In mtCamgaroo2, the worse effect of this reversible photoconversion is that not only the intensity of the signal...
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drops rapidly, but the residual fluorescence of the probe is insensitive to Ca\textsuperscript{2+}. The reason why this effect was not noted by Griesbeck et al. (16) is probably because of the very slow sampling rates used during their experiments. These problems, allied to the fact that mtCamgaroo-2 responds to Ca\textsuperscript{2+} increases only with an enhancement of fluorescence made us conclude that this probe is far from being an ideal Ca\textsuperscript{2+} indicator, at least for mitochondria.

Ratiometric pericam is the latest addition to the growing family of GFP-based Ca\textsuperscript{2+} indicators. Albeit not devoid of problems, at the moment this appears to be the less problematic probe for monitoring [Ca\textsuperscript{2+}]\textsubscript{m} at the single cell level. The advantages of PericamR are manifold. First, it exhibits very good targeting, even better than mtCamgaroo-2 (the residual nuclear signal is usually less than 10% of that in mitochondria). Second, it can be used in ratiometric mode. Third, even though some reversible photoconversion also occurs with this probe, it is far less than that observed with mtCamgaroo-2. Importantly, the residual fluorescent signal remains sensitive to Ca\textsuperscript{2+}.

Fourth, although we have not calculated accurately the matrix concentration of mtPericamR, the level of recombinant protein reached with other constructs rarely exceeds tenths of micromolar, whereas fluorescent probes usually reach concentrations of tens of micromolar (22). The main disadvantages that we have encountered with mtPericamR are its pH sensitivity and the effect of the local environment on the Ca\textsuperscript{2+} affinity. As to the former, it seems to be an intrinsic and unavoidable characteristic of all insertional mutants of GFP. As to the latter, the low affinity of mtPericamR for Ca\textsuperscript{2+}, once determined, turns out to be a serendipitous bonus that allows the investigation of a range of [Ca\textsuperscript{2+}] more appropriate for mitochondria.

Regarding the conclusions that can be drawn concerning the general response of mitochondria to Ca\textsuperscript{2+} mobilization in cells, the data obtained with mtPericamR at the single cell level are in good agreement with those obtained with mtCameleon and aequorin. There are, however, a few major discrepancies with data obtained in the same cell system with rhod-2. Overall, we note the following four points. First, in agreement with data obtained with aequorin and mtCameleon, the average increase in mitochondrial Ca\textsuperscript{2+} upon a histamine challenge is in the 10 \(\mu\text{m}\) range, higher than the \(\sim 5 \mu\text{m}\) calculated with rhod-2 (7).

The simplest explanation of this difference is the extra buffering capacity provided by the fluorescent indicator, although differences in the calibration procedure cannot be excluded. Second, consistent with data obtained with all the indicators, the peak mitochondrial Ca\textsuperscript{2+} is reached with a substantial delay (3–5 s) with respect to the nucleus and the cytoplasm. Third, in agreement with the results obtained with all other probes, also mtPericamR indicates that there is a substantial heterogeneity in [Ca\textsuperscript{2+}]\textsubscript{m} during InsP\textsubscript{3}-induced Ca\textsuperscript{2+} release. Fourth, unlike the data obtained with rhod-2 in HeLa cells, [Ca\textsuperscript{2+}]\textsubscript{m} appears to oscillate in synchrony with the cytosol, even if the mitochondrial Ca\textsuperscript{2+} oscillations are somehow attenuated. The group of Demaurex has also found mitochondrial Ca\textsuperscript{2+} oscillations in HeLa cells using mtCameleon\textsuperscript{3} and similar repetitive mitochondrial Ca\textsuperscript{2+} increases mirroring repetitive cytosolic Ca\textsuperscript{2+} changes have been reported in other model systems (8, 9, 23–25).

Last, but not least, we found no evidence for substantial mitochondrial desensitization upon repetitive histamine challenges. These observations are also in agreement with data obtained by us and others in permeabilized cells (26, 27). We note the discrepancy in relation to recently published data (7);

\textsuperscript{3} N. Demaurex, personal communication.
mizes the efficiency of information transfer between the two organelles. As such, it most likely plays a prominent role in second messenger signal transduction. The question now is to determine not only the nature of the molecular components that allow this structural interaction to occur, but also to understand their relationship to the Ca²⁺ release machinery of the ER.

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