

Mitochondrial Ca^{2+} Homeostasis in Intact Cells

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Abstract. Ca^{2+} is a key regulator not only of multiple cytosolic enzymes, but also of a variety of metabolic pathways occurring within the lumen of intracellular organelles. Until recently, no technique to selectively monitor the Ca^{2+} concentration within defined cellular compartments was available. We have recently proposed the use of molecularly engineered Ca^{2+} -sensitive photoproteins to obtain such a result and demonstrated the application of this methodology to the study of mitochondrial and nuclear Ca^{2+} dynamics. We here describe in more detail the use of chimeric recombinant aequorin targeted to the mitochondria. The technique can be applied with equivalent results to different cell models, transiently or permanently transfected. In all the cell types we analyzed, mitochondrial Ca^{2+} concentration ($[\text{Ca}^{2+}]_m$) increases rapidly and transiently upon stimulation with agonists coupled to InsP3 generation. We confirm that the high speed of mitochondrial Ca^{2+} accumulation with this type of stimuli depends on the generation of local gra-

dients of Ca^{2+} in the cytosol, close to the channels sensitive to InsP3. In fact, only activation of these channels, but not the simple release from internal stores, as that elicited by blocking the intracellular Ca^{2+} ATPases, results in a fast mitochondrial Ca^{2+} accumulation. We also provide evidence in favor of a microheterogeneity among mitochondria of the same cells, about 30% of them apparently sensing the microdomains of high cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_c$). The changes in $[\text{Ca}^{2+}]_m$ appear sufficiently large to induce a rapid activation of mitochondrial dehydrogenases, which can be followed by monitoring the level of NAD(P)H fluorescence. A general scheme can thus be envisaged by which the triggering of a plasma membrane receptor coupled to InsP3 generation raises the Ca^{2+} concentration both in the cytoplasm (thereby triggering energy-consuming processes, such as cell proliferation, motility, secretion, etc.) and in the mitochondria, where it activates the metabolic activity according to the increased cell needs.

THE development of specific probes for measuring the Ca^{2+} concentration in the cytoplasm of living cells (Tsien et al., 1982; Grynkiewicz et al., 1985) has led to major progress in the understanding of the mechanisms controlling cellular Ca^{2+} homeostasis and of the role played by this cation in the chain of events coupling membrane receptor stimulation to cell activation (Berridge and Irvine, 1989; Pietrobon et al., 1990; Meldolesi et al., 1990). We now know that, in a variety of cell types, stimuli as diverse as hormones and growth factors, cell-cell interaction and synaptic transmission induce rises of the cytosolic free calcium concentration ($[\text{Ca}^{2+}]_c$). The Ca^{2+} ions then bind to a number of effector proteins, which, in turn, modulate a variety of cellular processes, such as motility, secretion, enzyme activation, etc. Rises in $[\text{Ca}^{2+}]_c$ depend either on influx from the extracellular medium and/or release from intracellular stores. In both cases, Ca^{2+} channels, located in the plasma membrane or in the membrane of specialized intracellular compartments, mediate the entry of Ca^{2+} into the cytosol of stimulated cells. These channels, differentially ex-

pressed in the various cell types, are localized in defined portions of the cell, and are regulated by specific effectors (membrane potential, ligand binding, G proteins, etc.) (Pozzan et al., 1994). As a result, the Ca^{2+} signal exhibits a high level of spatio-temporal organization, the physiological significance of which, however, is still largely mysterious.

Up until recently, the role of mitochondria in intracellular Ca^{2+} homeostasis was somewhat controversial. Well before Ca^{2+} emerged as a ubiquitous second messenger, studies in isolated organelles showed that mitochondria are endowed with a complex mechanism which mediates the transport of Ca^{2+} across the ion-impermeable inner membrane (for a review see Nicholls and Akerman, 1982; Carafoli, 1987). Although the molecular nature of the carriers was (and still is) unknown, two pathways was identified: an electrogenic uniporter and two electroneutral exchangers, a $2\text{Na}^+/\text{Ca}^{2+}$ and a $2\text{H}^+/\text{Ca}^{2+}$ antiporter. The uniporter, which is specifically inhibited by Ruthenium red (RR)¹ and La^{3+} , tends to

1. *Abbreviations used in this paper:* BAEC, bovine aortic endothelial cells; cADPribose, cyclic ADP ribose; CS, citrate synthase; FCCP, *p*-trifluoromethoxyphenylhydrazine; InsP3, inositol-1,4,5-trisphosphate; KRB, Krebs-Ringer buffer; LDH, lactate dehydrogenase; RR, Ruthenium red; SERCA, sarco/endoplasmic reticulum Ca^{2+} ATPase; tBuBHQ, *t*-butyl-benzohydroquinone.

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distribute Ca^{2+} according to its electrochemical gradient and thus, in energized mitochondria, which maintain an internally negative electrical gradient, promotes accumulation of Ca^{2+} within the matrix. Electrochemical equilibrium (which, for a membrane potential of ~ 180 mV, would result in a matrix Ca^{2+} concentration six orders of magnitude higher than $[\text{Ca}^{2+}]_c$) is prevented by the activity of the electroneutral exchangers, which transport Ca^{2+} out of the matrix in exchange for H^+ or Na^+ . The high efficiency of mitochondrial Ca^{2+} accumulation *in vitro* led to the suggestion that mitochondria play a major role in cellular Ca^{2+} handling. This view was heavily challenged by a series of experimental results. First, direct measurement of intramitochondrial Ca^{2+} using electron probe microanalysis showed that, under physiological conditions, the Ca^{2+} content of mitochondria is low, ~ 0.5 mmol/l (Somlyo et al., 1985). Second, it was demonstrated that, in most mammalian cell types, $[\text{Ca}^{2+}]_c$ at rest is ~ 0.1 μM , and briefly reaches, upon stimulation, values around 1 μM , i.e., well below the affinity of the mitochondrial uniport (for a review see Pietrobon et al., 1990). Third, the ER, or some of its subcompartments, was identified as the intracellular source of rapidly mobilizable Ca^{2+} (Streb et al., 1983; Berridge and Irvine, 1989; Berridge, 1993; Pozzan et al., 1994). The complex Ca^{2+} transport systems of mitochondria thus appear to be best tuned for the fine modulation of the activity of the Ca^{2+} -sensitive mitochondrial dehydrogenases (McCormack et al., 1990, 1994). Although this view was largely accepted, direct evidence for the occurrence of significant $[\text{Ca}^{2+}]_m$ variations in intact cells was not obtained until very recently (Miyata et al., 1991; Rizzuto et al., 1992).

A deeper insight into these phenomena is now possible by the application to these organelles of the new approach for measuring Ca^{2+} concentrations within defined cellular compartments, recently developed in our laboratory. This method is based on the recombinant expression of chimeric Ca^{2+} -sensitive photoproteins, targeted to intracellular organelles (Rizzuto et al., 1992; Brini et al., 1993). Using this technique we showed that large changes of $[\text{Ca}^{2+}]_m$ occur upon stimulation of cells with specific agonists (Rizzuto et al., 1992, 1993; Rutter et al., 1993) and provided evidence for the existence of microdomains of high $[\text{Ca}^{2+}]_c$ close to mitochondria. In this article we describe in detail a variety of methodological and conceptual aspects of this new technique. We provide direct evidence for the localization of the chimeric photoprotein in the mitochondrial matrix and discuss the various conditions for expression and reconstitution of mitochondrial aequorin. We have also further analyzed the mechanisms and the functional effects of the $[\text{Ca}^{2+}]_m$ changes elicited by stimulation with agonists coupled to InsP_3 generation, demonstrating a marked heterogeneity in the mitochondrial response to $[\text{Ca}^{2+}]_c$ increases.

Materials and Methods

Materials

Tissue culture medium and complements were purchased from Technogenetics (Milan, Italy), DNA modification and restriction enzymes from Boehringer Mannheim GmbH, (Mannheim, Germany) and New England Biolabs (Beverly, MA), chemicals from Sigma Chemical Co. (St. Louis, MO), RNAzol BTM from Biotex Laboratories, Inc. (Houston, TX) and ra-

dioactive material from Amersham International (Amersham, Buckinghamshire, UK). Coelenterazine, cyclic ADP ribose (cADPRibose), and *t*-butyl-*l*-benzohydroquinone (tBuBHQ) were kind gifts of Drs. Y. Kishi (Harvard University, Cambridge, MA), A. Galione (Oxford University, Oxford, UK) and A. Benedetti (Siena University, Siena, Italy), respectively.

Cell Culture and Transfection

All cells (HeLa line and clones, the cell line 143B and clones derived from the L929 line) were grown in DMEM, supplemented with 10% FCS, in 75 cm² Falcon flasks. For the transfection, cells were seeded onto Corning Glass Works (Corning, NY) Petri dishes (9-cm diam) and grown to 50% confluence. At this stage, transfection was carried out according to a standard Ca^{2+} -phosphate procedure, using, for each plate, either 40 μg of mtAEQ/pMT2 (for transient expression) or 36 μg of mtAEQ/pMT2 + 4 μg of pSV2neo (for the selection of stable clones). In brief, for a 9 cm Petri dish the DNA (precipitated overnight in 70% ethanol) was resuspended in 450 μl of sterile TE (10 mM Tris, 1 mM EDTA, pH 8.0); after addition of 50 μl of 2.5 M CaCl_2 , the solution was added under vortexing to 500 μl of 2 \times HBS (280 mM NaCl, 50 mM Hepes, 1 mM Na_2HPO_4 , pH 7.12 at 25°C), and incubated for 30 min at room temperature. The final solution was then added to the Petri dish (containing 10 ml of complete growth medium). In transient expression experiments, 100 μl of the same solution were added to 1 ml of medium in each well of a 24-well plate, containing a 13-mm-diam round coverslip (with the same cell density) at the bottom. In both cases, after an overnight incubation (37°C, 5% CO_2 atmosphere), the medium was changed and the incubation prolonged. On the next day, the cells were used for aequorin measurement in transient expression experiments or, alternatively, selection with 0.8 mg/ml G418 was started. After approximately 2 wk, G418-resistant clones were picked from the dish with a sterile toothpick (with cotton at the extremities) and transferred to 24-well culture plates. When the cells filled the wells, they were passed onto 75-cm² culture flasks and analyzed for mtAEQ expression.

Northern Analysis

RNA was extracted from the 75-cm² flasks with RNAzolTMB (used according to the protocol of the producing company). RNA electrophoresis and blotting were performed as elsewhere described (Rizzuto et al., 1994), using the random prime-labeled (Feinberg and Vogelstein, 1983) aequorin cDNA (Inouye et al., 1985) as a probe.

Cell Fractionation

Cells of the clone MH75 grown to confluence were scraped in 5 ml of PBS from a large Petri dish (15-cm diam) with a rubber policeman cut at a low angle. After centrifugation at 4°C at 1,000 rpm (Heraeus Minifuge GL) and resuspension in 2 ml of 0.25 M sucrose, 10 mM Tris and 0.1 mM EDTA, pH 7.4 (resuspension buffer, RB), the cells were broken by 10 passages through a 22G1 1/4 needle fitted on a 5 ml plastic syringe. After centrifugation at 4°C at 2500 rpm (Minifuge GL; Heraeus-Amersil, Sayerville, NJ), the pellet (nuclei + unbroken cells) was discarded and the supernatant recentrifuged at 4°C at 10,000 rpm (SS34 rotor; Sorvall Instruments, Newton, CT). The pellet (mitochondria) was resuspended in 1 ml of RB and the supernatant recentrifuged at 4°C at 40,000 rpm (50Ti rotor; Beckman Instruments, Palo Alto, CA). The pellet (microsomes) was resuspended in 1 ml of RB and used, together with the mitochondrial fraction and the soluble supernatant, for the following analyses.

In Vitro Analyses

The activity of the cytosolic marker lactate dehydrogenases (LDH) and that of the mitochondrial marker citrate synthase (CS) were followed spectrophotometrically (Martin and Denton, 1970). LDH: wavelength = 340 nm, assay = 50 mM $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$, pH 7.5, 0.63 pyruvate, 200 μM NADH, initiate the reaction with the sample addition. CS: wavelength = 412 nm, assay = 80 mM Tris, pH 8.0, 0.1 mM acetyl-CoA, 0.1 DTNB, initiate the reaction with 0.5 mM oxaloacetate.

For aequorin reconstitution, RB was supplemented with 4 mM EDTA, 1 mM PMSF, 150 mM β -mercaptoethanol, and 5 μM coelenterazine. After overnight incubation at 4°C in the dark, light emission after aequorin discharge with 10 mM CaCl_2 was measured with a Packard Picolite luminometer. The total amount of reconstituted aequorin was extrapolated by the comparison with the Ca^{2+} -dependent light emission of known amounts of purified aequorin.

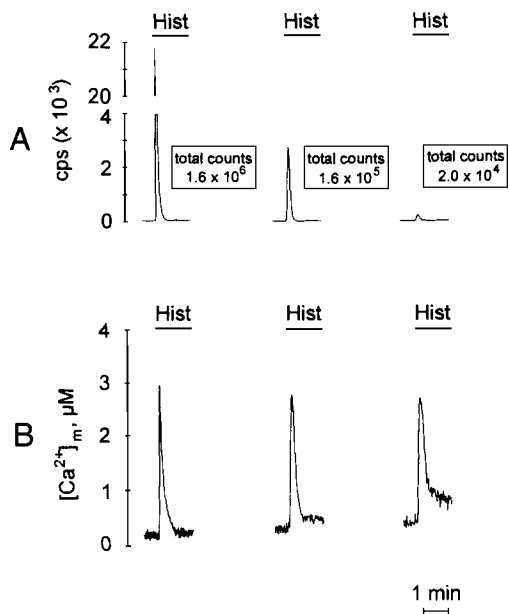


Figure 1. Light emission, and calculated $[Ca^{2+}]_m$ values, in three coverslips of MH75 cells varying substantially in cell number (and thus aequorin content). The cells were trypsinized, plated on round glass coverslips, and left in culture for 2 d. The reconstitution with coelenterazine, as well as the detection and calibration of the luminescence signal into $[Ca^{2+}]_m$ values, were carried out as described in Materials and Methods. Medium: KRB (125 mM NaCl, 5 mM KCl, 1 mM Na_3PO_4 , 1 mM $MgSO_4$, 5.5 mM glucose, 20 mM Hepes, pH 7.4, at 37°C), supplemented with 1 mM $CaCl_2$ (KRB/ Ca^{2+}). Panel A shows the light emission of mtAEQ (in cps); panel B shows the calculated $[Ca^{2+}]_m$ values. Where indicated, the cells were stimulated with 100 μ M histamine. The total number of counts (shown in the box on the right side of the luminescence data) was estimated by lysing the cells with a Ca^{2+} -rich, hyposmotic solution at the end of each experiment, thus discharging all unconsumed aequorin. These, and the following traces, are typical of 3–15 similar experiments, which gave the same results.

In Vivo $[Ca^{2+}]_m$ Measurements

For the aequorin-based $[Ca^{2+}]_m$ measurements, the cells were seeded onto 13-mm-diam glass coverslips a few days before the experiment. Aequorin was reconstituted, unless specified differently, by adding 5 μ M coelenterazine to the culture medium 2–3 h before the experiment. During the experiment, the cell monolayer was continuously perfused with modified Krebs-Ringer buffer (KRB): 125 mM NaCl, 5 mM KCl, 1 mM Na_3PO_4 , 1 mM $MgSO_4$, 5.5 mM glucose, 20 mM Hepes (pH 7.4 at 37°C), supplemented with either 1 mM $CaCl_2$ or 100 μ M EGTA, as specified in the figure legends. The various agonists and drugs were added to KRB, where indicated. In the experiments with permeabilized cells, the medium contained 140 mM KCl, 5 mM NaCl, 1 mM K_3PO_4 , 5.5 mM glucose, 2 mM $MgSO_4$, 1 mM ATP, 2 mM sodium succinate, 20 mM Hepes (pH 7.05 at 37°C), supplemented with either 50 μ M EGTA or Ca^{2+} -EGTA buffers (2 mM EGTA, free Ca^{2+} as indicated in the figure legend), prepared as previously described (Rizzuto et al., 1993). Light emission was measured in a purpose-built luminometer and calibrated in terms of $[Ca^{2+}]_m$, as described by Cobbold and Lee (1991). At the end of each experiment the cells were lysed by perfusing them with a hyposmotic medium containing only 10 mM $CaCl_2$, in order to expose all the aequorin contained by the cells to a high $[Ca^{2+}]_m$. The total amount of functional aequorin needs in fact to be known for converting luminescence data into $[Ca^{2+}]_m$ values, on the basis of the in vitro response curve of aequorin to Ca^{2+} (Moisescu and Ashley, 1977). The intensity of the light signal, however, does not affect per se the aequorin calibration. This is evident in Fig. 1, which presents the changes in aequorin luminescence (A), and the $[Ca^{2+}]_m$ values (B), elicited by

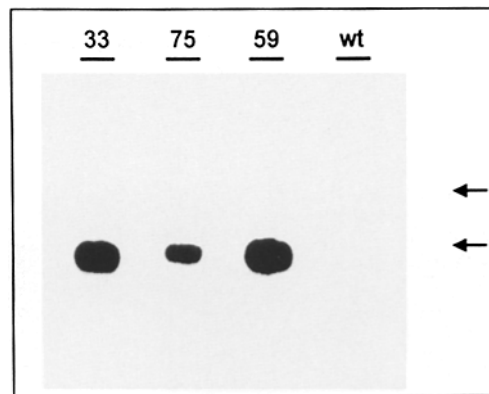


Figure 2. Northern analysis of mtAEQ expression of mitochondrial HeLa clones 33, 59, and 75. The RNA was extracted, electrophoresed, blotted, and hybridized as described in Materials and Methods. 30 μ g of total RNA were loaded in each lane. 33, 59, and 75 represent three HeLa clones expressing mtAEQ; *wt*, parental HeLa line. Arrows indicate the position of ribosomal RNAs.

histamine in three monolayers of MH75 differing substantially in cell density, and thus in total mtAEQ content. Despite the large differences in light output, the peak amplitudes of $[Ca^{2+}]_m$ are very similar, if not identical; however, when the total signal is low (traces on the right), the kinetics are somewhat smoothed and the estimates inaccurate at resting values, i.e., when the aequorin light output is hardly different from the background signal.

$[Ca^{2+}]_c$ was measured with fura-2 in monolayers of cells or at the single cell level, as described (Rizzuto et al., 1993). During the experiments, the cells were incubated with KRB and all additions were made in the same buffer.

NAD(P)H Measurements

For NAD(P)H measurements, the cells were plated onto rectangular coverslips (20-mm-long and 8-mm wide). After washing the cell monolayer with PBS, the coverslips were fitted in a fluorimeter cuvette containing KRB medium supplemented with 1 mM $CaCl_2$. All additions were made in the same medium. NAD(P)H autofluorescence was measured in a Perkin Elmer 650-40 fluorimeter (excitation 350, emission 460).

Results

Expression and Reconstitution of Mitochondrially Targeted Recombinant Aequorin

In our first report of mitochondrial targeting of the photoprotein aequorin (Rizzuto et al., 1992) we described the isolation of stably transfected clones from bovine aortic endothelial cells (BAEC). These clones, however, grew slowly and showed phenomena of end-stage differentiation and/or senescence, such as vacuolization, nuclear aberrations, etc. New clones were thus generated from the human cell line HeLa; HeLa cells were transfected with the mtAEQ expression plasmid (Rizzuto et al., 1992) in a 9:1 ratio with the selectable marker pSV2neo (Southern and Berg, 1982). 80 G418-resistant clones were screened by Northern blotting for the expression of mtAEQ mRNA. Most clones appeared positive, clones 33, 59, and 75 showing the most intense bands on the autoradiogram. Fig. 2 shows the results of a Northern analysis on the three selected clones (#33, 59, and 75). The size of the hybridizing band (\sim 2 kb, i.e., much

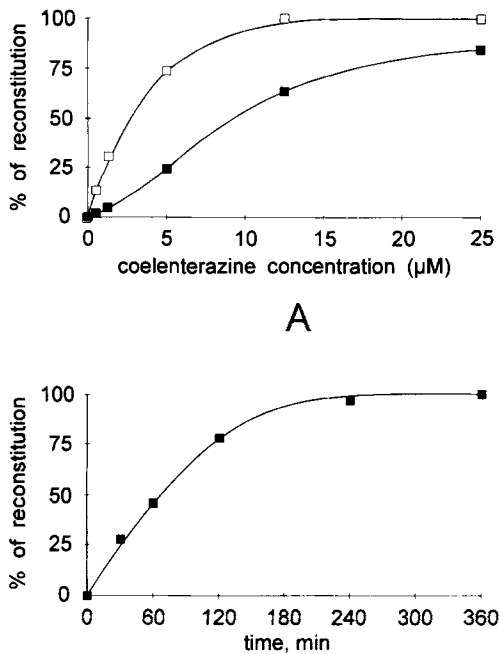


Figure 3. Time course and concentration dependency of mtAEQ reconstitution. Cells of the MH75 clone were seeded onto glass coverslips and left in culture for 2 d. *A* shows the dependency on coelenterazine concentration of aequorin reconstitution in MH75 cells. The cells were incubated for 2 h with the indicated concentration of coelenterazine; aequorin content was measured in the aequorin detection system by lysing the cells via perfusion with a Ca²⁺-rich hyposmotic solution (10 mM CaCl₂ in H₂O). Open and closed symbols refer, respectively, to reconstitution in KRB/Ca²⁺ and in DMEM + 10% FCS. *B* shows the time course of aequorin reconstitution in MH75 coverslips incubated with 5 μM coelenterazine in DMEM + 10% FCS. At the indicated times, the amount of reconstituted aequorin was estimated as in *A* and expressed as percentage of maximal reconstitution at this coelenterazine concentration. In both panels, the data points represent the mean of three independent experiments.

larger than that predicted for the mtAEQ mRNA, ~0.8 kb) suggests that, although a typical polyadenylation site AATAAA is present in the 3' noncoding region of the aequorin cDNA, in vivo polyadenylation occurs at the SV-40 polyA site of the vector, downstream of the 1-kb dehydrofolate reductase gene. As expected, this large 3' noncoding region did not prevent the efficient synthesis of the recombinant protein. In the three clones there was a good correlation between the mRNA levels and the amount of recombinant protein, as deduced by the in vitro reconstitution of functional aequorin after cell lysis (not shown). The data presented below refer mostly to clone 75 (denominated mitochondrial HeLa clone 75, or MH75), but similar data were obtained with the other clones.

Recombinant apoaequorin, as produced by transfected cells, is inactive and can be reconstituted in functional chemiluminescent protein by adding the prosthetic group coelenterazine to the culture medium of intact cells (Rizzuto et al., 1992, 1993). Fig. 3 *a* shows the dependency on coelenterazine concentration of functional mtAEQ reconstitution in intact cells. Maximal reconstitution, in KRB without serum, could be obtained with ~10 μM coelenterazine. The concentration of coelenterazine necessary for obtaining the

Table I. Subcellular Distribution of mtAEQ and Marker Enzymes

	Percent of Total		
	Lactate dehydrogenase	Citrate synthase	Aequorin
Fraction I (mitochondria)	5	98.5	97.5
Fraction II (microsomes)	9	0.5	0.5
Fraction III (cytosol)	86	1	2

Cell fractionation of MH75 cells, measurement of the activity of the marker enzymes and reconstitution in vitro and measurement of aequorin were performed as described in the Materials and Methods section. Numbers indicate the relative distribution of the three proteins (expressed as percent of total enzymatic activity and aequorin content) in the three subcellular fractions.

maximum light emission increased slightly at higher cell densities (not shown). In the presence of serum, reconstitution efficiency was reduced, presumably due to nonspecific binding of coelenterazine to serum proteins. However, given the limited supply of coelenterazine and the sensitivity of the system, a good compromise was the addition of 5 μM coelenterazine to the complete growth medium (DMEM + 10% FCS), at 37°C and in 5% CO₂ atmosphere (Fig. 3 *a*); these conditions were used in all the following experiments. Fig. 3 *b* shows the time course of aequorin reconstitution, as obtained by incubating intact HeLa cells with 5 μM coelenterazine at 37°C in DMEM (+10% FCS). Good luminescence signals were observed after incubations as short as 30 min, though maximal reconstitution for those conditions required about 2–3 h. Longer incubations resulted in no further increase (but rather in a decrease) of total luminescence.

Subcellular Distribution of Recombinant Aequorin

The subcellular localization of mtAEQ was next investigated. In agreement with our previous data in the endothelial clone M4 (Rizzuto et al., 1992), treatment of MH75 cells with digitonin (a detergent which preferentially interacts with cholesterol containing membranes) caused the release in the supernatant of most of the cytosolic marker enzyme LDH, while >90% of mtAEQ remained associated with the pellet (not shown). This experiment however only demonstrates that mtAEQ is not soluble in the cytosol, but does not prove that the recombinant protein is sequestered inside mitochondria.

A more accurate subcellular fractionation was thus carried out. Several homogenization procedures were attempted, i.e., Potter-Elvehjem homogenizer, whirly mixer, etc., but all of them appeared too drastic for HeLa cells and a large amount of a mitochondrial matrix enzyme marker (CS) was recovered in the soluble supernatant (together with mtAEQ). The procedure which gave the most satisfactory result was the mechanical disruption of the cells obtained by passage of the cell suspension through a stainless steel needle, as described in Materials and Methods. This procedure did not give a high yield of cell disruption (~50% of the cells remained intact), but the release of the mitochondrial matrix enzyme marker into the soluble supernatant was negligible.

As shown in Table I, after discarding unbroken cells and nuclei, almost all CS and mtAEQ were recovered in fraction

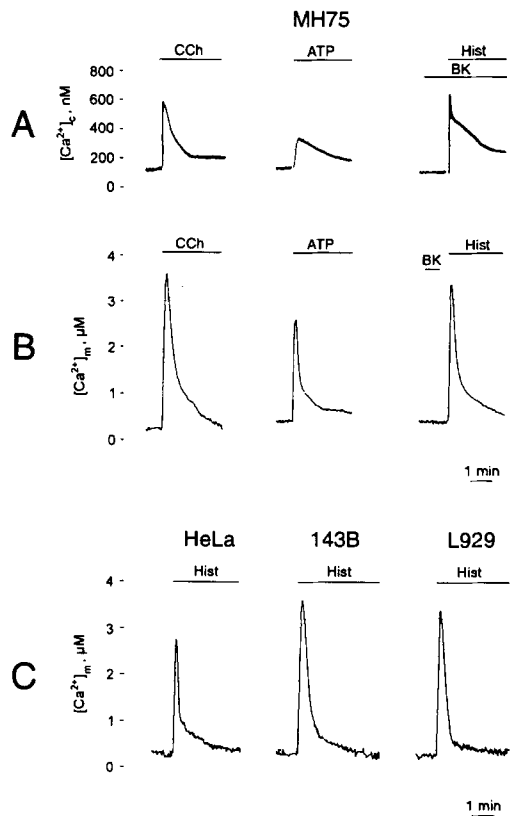


Figure 4. $[Ca^{2+}]_e$ and $[Ca^{2+}]_i$ responses in MH75 cells upon stimulation with different agonists; $[Ca^{2+}]_i$ responses in different cell types. *A* and *B* show the $[Ca^{2+}]_e$ and $[Ca^{2+}]_i$ responses of MH75 cells to a number of agonists. Cells of the MH75 clone were seeded onto glass coverslips and left in culture for 2 d. The coverslips were incubated in KRB/ Ca^{2+} medium. Where indicated, the cells were stimulated with 50 μ M carbachol (CCh), 100 μ M ATP, 500 μ M bradykinin (BK) and 100 μ M histamine (Hist), added to the same medium. Aequorin luminescence was measured and calibrated into $[Ca^{2+}]_i$ values as in Fig. 1. Fura-2 measurements were carried out on monolayers of MH75 cells as described in Materials and Methods. *C* shows the $[Ca^{2+}]_i$ responses to histamine of three different cell lines or clones. Experimental conditions as in Fig. 1. *HeLa*, transiently transfected parental HeLa cell line; *143B*, transiently transfected human osteosarcoma cell line 143B; *L929*, cellular clone derived from the mouse fibroblast cell line L929, stably expressing mtAEQ. Where indicated, the cells, perfused with KRB, were challenged with 100 μ M histamine.

I (mitochondrial fraction), while only trace amounts of these two proteins were recovered in fractions II (microsomes) and III (cytosol). Conversely, LDH was largely associated with the cytosolic fraction. Although these results provide strong evidence in favor of the mitochondrial localization of mtAEQ, they do not discriminate whether mtAEQ is located in the matrix, in the outer or inner membrane or in the intermembrane space. The possibility that mtAEQ is membrane bound or within the intermembrane space can be excluded on the basis that: (a) the cDNA coding for mtAEQ does not contain hydrophobic stretches which could anchor the recombinant protein to the membrane; (b) disruption of the outer membrane by hypotonic treatment of digitonin permeabilized cells resulted in marginal release (<10%) of mtAEQ into the supernatant; and (c) upon freeze thawing of the mitochondrial fraction most of the aequorin (>75%) was

recovered in the soluble fraction. Further functional evidence for the matrix localization of mtAEQ is provided by the experiment presented in Fig. 5.

Large Increases in $[Ca^{2+}]_m$ Parallel Agonist-induced $[Ca^{2+}]_e$ Changes in a Variety of Cell Types

HeLa cells of clone MH75 were challenged with agonists acting via the inositol-1,4,5-triphosphate (InsP₃)/ Ca^{2+} pathway and the changes in mtAEQ luminescence monitored in parallel with the $[Ca^{2+}]_e$ variations (measured with fura-2). The cells responded with large $[Ca^{2+}]_e$ increases to carbachol, histamine and ATP, but not to bradykinin (Fig. 4 *A*); accordingly, also $[Ca^{2+}]_m$ was increased by carbachol, histamine, and ATP, but not by bradykinin (Fig. 4 *B*). Similar results were obtained in the other clones, except that clone 59 responded poorly to ATP, but showed a good response to bradykinin, both in terms of $[Ca^{2+}]_e$ and of mtAEQ luminescence (not shown).

The rapid increase of $[Ca^{2+}]_m$ upon stimulation with agonists coupled to InsP₃ generation (~ 0.5 μ M/s) is not specific to these clones, nor to the previously described endothelial clone M4 (Rizzuto et al., 1992). This is evident from Fig. 4 *C*, where different cell types have been analyzed. The left and middle traces refer, respectively, to the parental HeLa line and to the cell line 143B, originated from a human osteosarcoma, transiently transfected with the mtAEQ/pMT2 plasmid. The trace on the right refers to a cellular clone isolated from the transfection of the mouse fibroblast cell line L929 (Van Haesebroeck, B., manuscript in preparation). In all cases, the stimulation of the cells with an agonist acting via the InsP₃/ Ca^{2+} pathway induced a rapid rise in $[Ca^{2+}]_m$, which reached values of 2–4 μ M and rapidly returned close to basal values. It is interesting to note that targeted recombinant aequorin can be transiently expressed at high levels, thereby indicating that this technique can be used also when the isolation of stably transfected clones is not possible. In fact, high transient expression of mtAEQ has been recently obtained in primary cultures of neurons and myocytes (Rizzuto, R., manuscript in preparation).

Mitochondrial Ca^{2+} Uptake: Kinetics and Sensitivity to Mitochondrial Drugs

Mitochondrial Ca^{2+} accumulation depends on the activity of an electrogenic carrier, that tends to equilibrate Ca^{2+} with its electrochemical gradient. When, following cell stimulation, extramitochondrial $[Ca^{2+}]_e$ increases, a rapid mitochondrial Ca^{2+} uptake can be observed. Mitochondrial Ca^{2+} uptake can be inhibited in two ways, i.e., either by directly blocking the influx mechanism or by collapsing the thermodynamic driving force. On the latter aspect, we have previously shown that the mitochondrial uncoupler *p*-trifluoromethoxyphenylhydrazine (FCCP) dramatically reduces the agonist-induced light emission of mtAEQ-transfected endothelial cells (Rizzuto et al., 1992). Fig. 5 *A* shows the effect of FCCP on the agonist-induced $[Ca^{2+}]_m$ transients of MH75 cells. The traces on the left refer to control cells, those on the right to FCCP-treated cells. The addition of FCCP caused a small $[Ca^{2+}]_e$ increase and did not reduce significantly the $[Ca^{2+}]_e$ peak induced by histamine (traces *a* and *b*). In contrast, the addition of FCCP caused a small, but clear, reduction in the resting value of $[Ca^{2+}]_m$ and

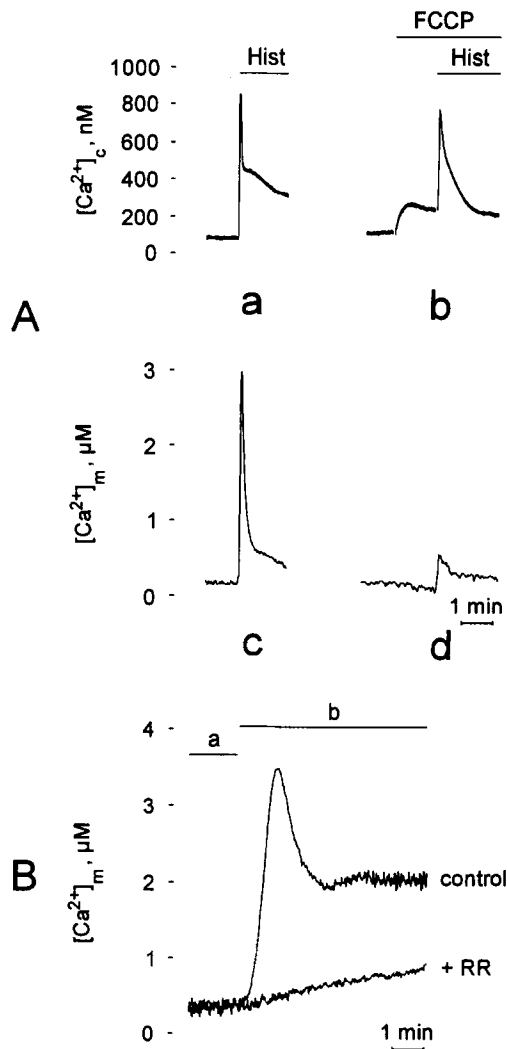


Figure 5. Effect of mitochondrial inhibitors on $[Ca^{2+}]_m$ responses of MH75 cells. Aequorin light emission of MH75 cells was measured and calibrated as described in Fig. 1. **A** shows the measurements of $[Ca^{2+}]_m$ and $[Ca^{2+}]_c$ in intact cells. Where indicated, the cells, perfused with KRB/ Ca^{2+} , were challenged with 100 μ M histamine (*Hist*) and/or 2 μ M FCCP. Other conditions as in Fig. 1. **B** shows the $[Ca^{2+}]_m$ measurements in permeabilized cells. Medium (buffer a): 140 mM KCl, 5 mM NaCl, 1 mM K_3PO_4 , 5.5 mM glucose, 2 mM $MgSO_4$, 1 mM ATP, 2 mM sodium succinate, 20 mM Hepes (pH 7.05 at 37°C), 50 μ M EGTA. Before recording, the cells were permeabilized by a 2-min incubation with 100 μ M digitonin (added to the same buffer). Where indicated (buffer b), the medium was changed to an EGTA-based Ca^{2+} buffered saline (2 mM total EGTA, 2 μ M free Ca^{2+} , all other constituents as in buffer a). In the indicated trace (+ RR), the medium was supplemented with 4 μ M Ruthenium red.

drastically reduced the $[Ca^{2+}]_m$ increase evoked by the addition of histamine (traces *c* and *d*), in agreement with the data on endothelial cells. Similar data were obtained in transiently transfected primary cultures of myocytes and neurons, indicating an efficient targeting of mtAEQ to the mitochondrial matrix also in cell types which differ significantly in their repertoire of Ca^{2+} stores and channels.

Other mitochondrial inhibitors, such as RR, a blocker of

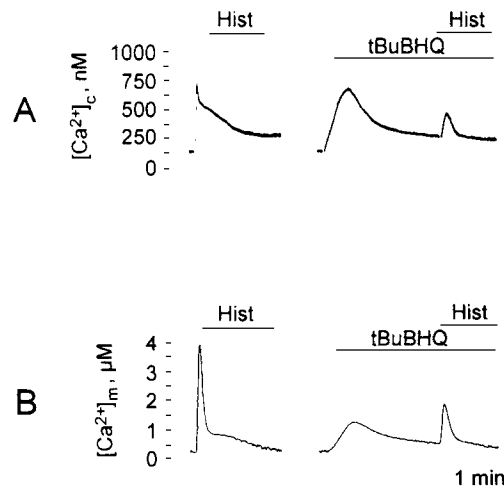


Figure 6. The effects on $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ of two agents (tBuBHQ and histamine) releasing Ca^{2+} from intracellular stores by different mechanisms (membrane leak and channels opening, respectively) in MH75 cells. The upper and lower panel show the $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ measurements, respectively. All conditions as in Fig. 4. Where indicated, the cells were perfused with the same medium (KRB/ Ca^{2+}), supplemented with 100 μ M histamine (*Hist*) and/or 100 μ M tBuBHQ. tBuBHQ was preferred to thapsigargin as SERCA blocker, because the latter compound tends to adhere to the plastic tubing of the perfusion system. Indeed, in cells transfected with cytosolic aequorin, no effect on $[Ca^{2+}]_c$ was detected upon treatment with thapsigargin, while tBuBHQ induced a $[Ca^{2+}]_c$ rise comparable to that measured in cuvette with fura-2.

the Ca^{2+} uniport, rotenone, an inhibitor of the respiratory chain and oligomycin, an inhibitor of the H^+ ATPase, were also tested. Neither rotenone nor oligomycin, when applied alone, induced any significant change of the $[Ca^{2+}]_m$ increases caused by receptor stimulation. This result was expected since, in intact cells, a mitochondrial negative potential can be maintained by either the respiratory chain or the H^+ ATPase, and thus inhibition of only one of the two should not depolarize the mitochondria. In fact, the two drugs together caused a significant reduction of agonist-dependent $[Ca^{2+}]_m$ increases, though never as efficiently as FCCP (data not shown). As to RR, up to 50 μ M it had no effect on $[Ca^{2+}]_m$ changes in intact HeLa cells, in contrast with results obtained with cardiac myocytes (Miyata et al., 1991).

One explanation of this discrepancy could be the impermeability of the plasma membrane of HeLa cells to this compound. In order to verify this possibility, the effect of RR was tested after permeabilization of the plasma membrane with digitonin (Fig. 5 *B*). In this experiment, the cells were initially perfused with Ca^{2+} -free EGTA containing buffer (free $Ca^{2+} < 10^{-8}$ M) and then treated with digitonin, which makes large holes in the plasma membrane while leaving the organelles in situ. Addition of digitonin resulted in a variable, rapid and transient increase in $[Ca^{2+}]_m$ (not shown). This effect presumably depends on a digitonin-induced discharge of InsP3-sensitive Ca^{2+} stores. In fact, if an InsP3 producing stimulus (e.g., histamine) was added before digitonin, the detergent caused only a marginal increase in $[Ca^{2+}]_m$. After the digitonin-induced spike, $[Ca^{2+}]_m$ re-

mained constant until the medium $[Ca^{2+}]_e$ was raised to 2 μM (EGTA buffered). Under these conditions, $[Ca^{2+}]_m$ first increased up to about 3.5 μM , followed by a return and leveling off around 2 μM . This value remained unchanged for several min, i.e., as long as the $[Ca^{2+}]_e$ in the perfusing buffer was kept constant. In the presence of RR, the $[Ca^{2+}]_m$ increase was drastically reduced both in rate and in amplitude, but not totally abolished. The residual uptake was not affected by higher doses of the inhibitor (up to 20 μM) and is probably attributable to reversal of the activity of the $2Na^+(H^+)/Ca^{2+}$ antiporter. Taken together, the experiments presented in Fig. 5 conclusively demonstrate that recombinant aequorin is contained in the mitochondrial matrix (a RR- and FCCP-sensitive compartment) and not in the intermembrane space, which is known to be in rapid equilibrium with the external medium.

Mitochondrial Ca^{2+} Accumulation Depends on the Rapid Release of Ca^{2+} from Intracellular Ca^{2+} Stores

We have recently shown (Rizzuto et al., 1993) that the high rate of $[Ca^{2+}]_m$ increase observed in intact cells stimulated with agonists linked to InsP3 formation (see for example Fig. 4 B) largely exceeds the predictions based on the average changes of $[Ca^{2+}]_e$. We attributed this phenomenon to the generation of local domains of high $[Ca^{2+}]_e$, close to InsP3-gated channels, which are sensed by neighboring mitochondria. The data presented in Figs. 6 and 7 confirm and extend

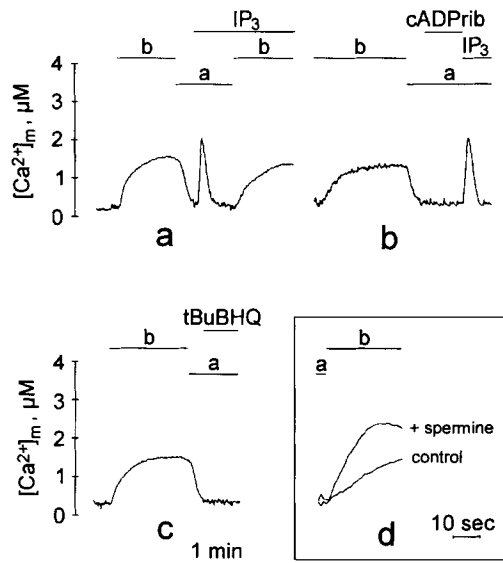


Figure 7. $[Ca^{2+}]_m$ measurements in permeabilized MH75 cells. All conditions as in Fig. 5 B. Before recording, the cells were permeabilized by a 2-min incubation with digitonin, added to buffer a (see Fig. 5). Where indicated (buffer b), the Ca^{2+} -free KCl buffer (buffer a) was substituted with an EGTA-based Ca^{2+} buffered saline (2 mM total EGTA, 1.5 μM free Ca^{2+} , all other constituents as in buffer a). Where indicated, the cells were perfused with 5 μM InsP3 (IP_3), 10 μM cADPribose ($cADPrib$), and/or 100 μM tBuBHQ. In d, in the indicated trace (+ spermine) the medium was supplemented with 0.5 mM spermine; the time scale of this panel is expanded in order to better appreciate the differences in the rates of $[Ca^{2+}]_m$ increase.

these observations. Fig. 6 compares the effect on $[Ca^{2+}]_m$ of the Ca^{2+} release from intracellular stores via two different mechanisms, i.e., through InsP3-gated channels and by the "membrane leak" which follows the blocking of the Ca^{2+} ATPase with specific inhibitors, such as tBuBHQ (Kass et al., 1989). The upper panel shows the $[Ca^{2+}]_e$ measurement with fura-2, the lower panel the $[Ca^{2+}]_m$ kinetics. The $[Ca^{2+}]_e$ rise caused by tBuBHQ was similar in size, but markedly slower than that induced by the InsP3 generating agonist, while the $[Ca^{2+}]_m$ rise was significantly larger and faster in the latter case. Addition of histamine after a 5-min incubation with tBuBHQ induced a $[Ca^{2+}]_e$ rise smaller than that induced by the blockade of the Ca^{2+} ATPase; also in this case, the $[Ca^{2+}]_m$ rise caused by histamine was larger and faster than that induced by tBuBHQ, indicating that even small increases of $[Ca^{2+}]_e$, if generated through InsP3 channels, are particularly effective at causing $[Ca^{2+}]_m$ rises.

Fig. 7 shows a series of experiments on permeabilized cells. In agreement with the data of Fig. 6, only the rapid release of Ca^{2+} evoked by InsP3 caused a fast increase of $[Ca^{2+}]_m$ (Fig. 7 a), while that induced by tBuBHQ determined a negligible accumulation of Ca^{2+} in the mitochondrial matrix (Fig. 7 c). It could be argued that InsP3, by acting on an unknown mitochondrial receptor, could increase dramatically the affinity of the mitochondrial Ca^{2+} uptake mechanism. This possibility is ruled out by the experiment presented in Fig. 7 a. In permeabilized cells perfused with 1 μM free $[Ca^{2+}]_e$, the rate of mitochondrial $[Ca^{2+}]_m$ rise was similar whether InsP3 was present or not in the medium. InsP3 is not the only agent which has been proposed to act as an intracellular mediator of Ca^{2+} release. In particular, cADPribose has been shown to mobilize Ca^{2+} from intracellular stores in sea urchin eggs (Galione et al., 1993) and islet β -cells (Takasawa et al., 1993; but see also Islam et al., 1993); Fig. 7 b shows, however, that cADPribose did not cause any appreciable increase in $[Ca^{2+}]_m$ nor a potentiation of the InsP3 effect, under conditions in which the latter compound was highly effective. This result is consistent with the observation that in this HeLa cell clone the other pharmacological activator of the ryanodine receptor, caffeine, was without effect on $[Ca^{2+}]_e$ or $[Ca^{2+}]_m$ (data not shown), implying that these cells do not express significant levels of this intracellular Ca^{2+} channel. Of interest, on the other hand, is the observation that in permeabilized cells the polyamine spermine, at concentrations close to those found in the cytoplasm of living cells (Pegg and McCann, 1982), causes a 2.5-fold enhancement of the rate of $[Ca^{2+}]_m$ increase (Fig. 7 d), in agreement with previous observations that this compound may act as a positive effector of the mitochondrial Ca^{2+} uniporter (Nicchitta and Williamson, 1984; McCormack et al., 1989). We previously reported that, for the same increase in mean $[Ca^{2+}]_e$, Ca^{2+} release from stores caused a four to fivefold faster $[Ca^{2+}]_m$ increase than Ca^{2+} influx through the plasma membrane (Rizzuto et al., 1993). Even in the latter case, we noticed, however, that the rate of $[Ca^{2+}]_m$ rise was about twofold faster than predicted from the rate of $[Ca^{2+}]_e$ increase measured in permeabilized cells exposed to the same extramitochondrial $[Ca^{2+}]_e$ (Rizzuto et al., 1993). In the case of Ca^{2+} influx, the positive effect of spermine on mitochondrial Ca^{2+} uptake may account for the small discrepancy between the rate of Ca^{2+} accumulation in intact and permeabilized cells (Rizzuto et al., 1993).

Clearly this explanation is not sufficient in the case of Ca^{2+} mobilization.

After Accumulation, Ca^{2+} Ions Are Released from Mitochondria through the Ca^{2+} Electroneutral Exchanger

The data so far presented (for example see Fig. 4) demonstrate that, after the initial spike, $[\text{Ca}^{2+}]_m$ returned rapidly close to basal levels. It should be noted that the leveling off of $[\text{Ca}^{2+}]_m$ was quite variable between cell batches. In some cases the steady state was indistinguishable from the resting level, in others it was slightly, but clearly, elevated. The reason for this variability is presently unknown. The question arises as to whether the rapid decrease of $[\text{Ca}^{2+}]_m$ is due to slow intramitochondrial Ca^{2+} buffering or to Ca^{2+} extrusion via the antiporters. We directly addressed this issue in the experiment presented in Fig. 8, which shows the effect of the antiporter inhibitor diltiazem (Vaghy et al., 1982; Rizzuto et al., 1987). The cells were continuously perfused with 100 μM diltiazem, which caused per se a slight increase in basal $[\text{Ca}^{2+}]_m$. Upon stimulation with histamine, diltiazem had no effect on the initial rate of $[\text{Ca}^{2+}]_m$ increase, but caused a small increase in the peak $[\text{Ca}^{2+}]_m$ and a very marked reduction ($\sim 40\%$) in the rate of $[\text{Ca}^{2+}]_m$ decrease. Considering that, even in isolated mitochondria, the inhibition of the antiporters (the $2\text{H}^+/\text{Ca}^{2+}$ in particular) by this concentration of diltiazem is not complete (Rizzuto et al., 1987), the experiment suggests that in vivo the Ca^{2+} electroneutral exchangers of the inner mitochondrial membrane play a major role in allowing the return of $[\text{Ca}^{2+}]_m$ to submicromolar values, thereby preventing excessive Ca^{2+} accumulation and thus mitochondrial damage.

Mitochondrial Ca^{2+} Accumulation Activates the NADH-linked Dehydrogenases

The large effect on $[\text{Ca}^{2+}]_m$ of InsP_3 -induced Ca^{2+} release from intracellular stores provides a rational explanation for the observation that relatively small increases in $[\text{Ca}^{2+}]_c$ result in significant increases in mitochondrial NAD(P)H (see for example Pralong et al., 1992; Duchen and Biscoe, 1992). The experiment presented in Fig. 9 demonstrates that indeed there is a good temporal correlation between the $[\text{Ca}^{2+}]_m$ rise (Fig. 9 a) and the increase in the levels of NAD(P)H (Fig. 9 c), which presumably reflects the activation of matrix dehydrogenases. However, $[\text{Ca}^{2+}]_m$ returned to basal values earlier than NAD(P)H, thus indicating either a relatively slow consumption of NAD(P)H or/and a delayed inactivation of the dehydrogenases. The increase in fluorescence was entirely attributable to mitochondrial, and not cytosolic, NAD(P)H reduction. In fact, if rotenone (a potent inhibitor of mitochondrial NADH dehydrogenase) was added before histamine, it caused a large increase in fluorescence, but no further effect was observed with the receptor agonist (Fig. 9 d).

Is the $[\text{Ca}^{2+}]_m$ Response Heterogeneous in the Mitochondrial Population?

The rapid rise in $[\text{Ca}^{2+}]_m$ caused in intact cells by agonists linked to InsP_3 production, or in permeabilized cells by InsP_3 itself, appears to be due to the formation of microdomains of high $[\text{Ca}^{2+}]_c$ sensed by neighboring mitochondria.

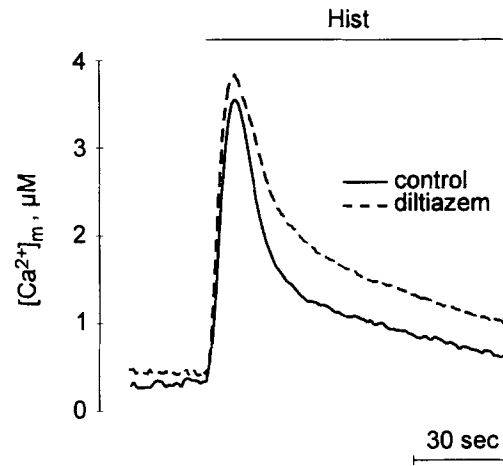


Figure 8. Effect of diltiazem on histamine-induced $[\text{Ca}^{2+}]_m$ transients in MH75 cells. All conditions as in Fig. 4. Where indicated, 100 μM histamine was added to the perfusion medium. In the experiment presented as a dotted trace, the medium was supplemented with 100 μM diltiazem.

An important physiological question is whether all mitochondria, or a subpopulation of them, share this condition. A direct answer to this question by analyzing individual mitochondria is presently beyond the limits of the available measuring apparatus. The problem was thus approached indirectly taking advantage of the peculiarity of the Ca^{2+} -triggered luminescence of aequorin. The rationale of the approach is briefly discussed below.

Aequorin light emission is a Ca^{2+} -dependent irreversible reaction: in the course of an experiment, a fraction of the photoprotein pool is "consumed," which is proportional to the amplitude and duration of the $[\text{Ca}^{2+}]_m$ to which aequorin is exposed. Thus, if the cells are challenged with two equally effective stimuli, the light output evoked by the second stimulus is expected to be reduced, when compared to the first,

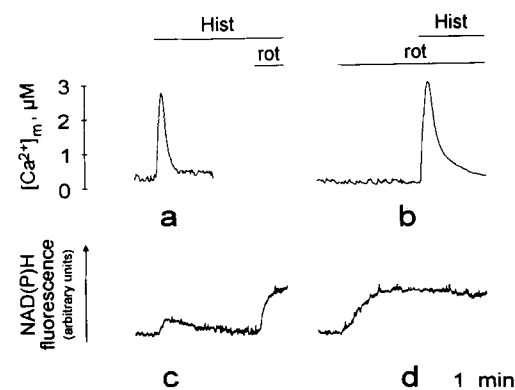


Figure 9. Histamine-induced changes in $[\text{Ca}^{2+}]_m$ and NAD(P)H fluorescence in MH75 cells. Experimental conditions, and incubation medium, as in Fig. 4. The upper and lower traces show the changes in $[\text{Ca}^{2+}]_m$ and NAD(P)H fluorescence, respectively. mtAEQ light emission was measured and calibrated as in Fig. 1; NAD(P)H fluorescence was measured as specified in the Materials and Methods section. Where indicated, the cells were challenged with 100 μM histamine (*Hist*) and/or 2 μM rotenone (*rot*).

proportionally to the amount of aequorin consumed by the first stimulus. The calculated $[Ca^{2+}]_m$, however, should be identical. Obviously this is true only if all mitochondria (and all cells) respond homogeneously. Conversely, if only a subpopulation of mitochondria (or of cells) respond to the stimuli, this subset will consume a substantial part of its aequorin content during the first challenge. In the latter case, the light emitted in the second stimulation will be drastically reduced and the calculated $[Ca^{2+}]_m$ artefactually underestimated.

The experiments presented in Fig. 10 were carried out to discriminate between these two possibilities. MH75 cells (loaded with fura-2) were stimulated in sequence with two agonists (carbachol and histamine) and analyzed individually by digital image microscopy for $[Ca^{2+}]_c$ responses. We preferred the use of two different stimuli to the double application of the same agonist because we observed a variable and heterogeneous homodesensitization which could complicate the interpretation of the results. The kinetics of $[Ca^{2+}]_c$ increases in three individual cells, typical of over 200 cells, is presented in Fig. 10, *a* and *b*. The $[Ca^{2+}]_c$ response was: (*a*) qualitatively quite homogeneous, i.e., all cells responded with a higher rise to histamine than to carbachol; (*b*) no cross-desensitization was observed; and (*c*) the amplitude and the kinetics of the $[Ca^{2+}]_c$ changes appeared independent of the order in which the agonists were added. Fig. 10, *c* and *d* show the mtAEQ photon emission kinetics, with the same protocol. In Fig. 10 *c*, histamine-induced light emission was $\sim 20,000$ cps at the peak, and consumed about 30% of the total aequorin content. The

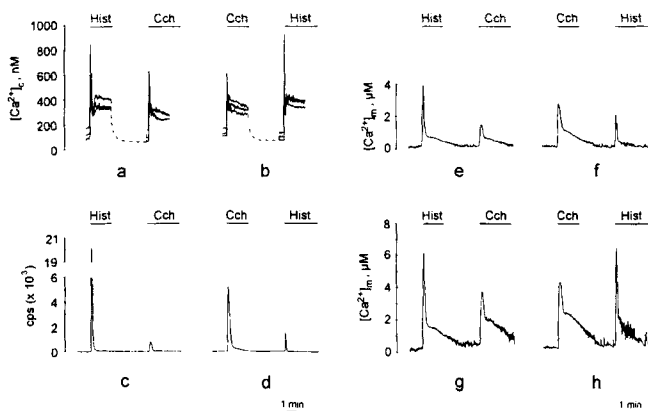


Figure 10. $[Ca^{2+}]_c$ changes, mtAEQ light emission and calibration into $[Ca^{2+}]_m$ values with two different procedures upon repetitive stimulation with InsP3-generating agonists. Experimental conditions, and perfusion buffer, as in Fig. 4. Traces *a* and *b* refer to single cell fura-2 recordings of three typical cells. The fura-2 signal was calibrated as specified in the Materials and Methods section. Where indicated, the cells were challenged with $100 \mu M$ histamine (*Hist*) and $50 \mu M$ carbachol (*CCh*); the dotted line shows the interruption of the recording during the wash-out of the first agonist. Traces *c* and *d* show the mtAEQ light emission in a parallel batch of cells. Traces *e* and *f*, and traces *g* and *h* show the conversion of aequorin luminescence into $[Ca^{2+}]_m$ values as obtained with two calibration procedures. Aequorin content was estimated as in Fig. 1. In traces *e* and *f*, the whole aequorin content was taken into account for calibration; in traces *g* and *h*, calibration was based only on the aequorin content of the highly responsive mitochondrial pool (see text).

mtAEQ light emission caused by carbachol, added 5 min later, was ~ 800 cps at the peak and total consumed aequorin was about 5% of the remaining photoprotein. However, if carbachol was added first (Fig. 10 *d*) light emission was as large as 5,000 cps and the overall aequorin consumption was about 28% of the total. The signal elicited by histamine, after carbachol, was drastically reduced, when compared to that of Fig. 10 *a*, both in terms of peak amplitude and total consumption. If a third stimulus was applied (second challenging with histamine or carbachol or first stimulation with ATP), a further drastic reduction of aequorin light emission was observed: the integral of the third peak corresponded to $<10\%$ of that elicited by the same stimulus in untreated cells (data not shown).

These experiments appear consistent with the hypothesis that only a subpopulation of mitochondria responds with a rapid and large rise of $[Ca^{2+}]_m$ to agonist stimulation, and thus consumes a large fraction of its aequorin content, while in the rest of mitochondria the $[Ca^{2+}]_m$ rise is small, and thus the aequorin pool is minimally consumed. Out of five experiments of this type, we calculated that the highly responsive mitochondria account for $\sim 30\%$ of the total cellular pool (see discussion). Fig. 10 (*e-h*) presents the conversion of the data of Fig. 10 (*c* and *d*) into $[Ca^{2+}]_m$ values, as obtained with either a normal calibration procedure (i.e., considering the whole aequorin pool, Fig. 10, *e* and *f*) or with the assumption that the aequorin content of the highly responsive subpopulation represents $\sim 30\%$ of total (Fig. 10, *g* and *h*). It is interesting to note that, in the second case, the amplitudes of the $[Ca^{2+}]_m$ changes induced by histamine and carbachol are independent of the order of stimulus addition.

An alternative explanation of these results would be that the first mitochondrial Ca^{2+} transient induces a long-term modification (e.g., opening of the mitochondrial pore, change in membrane potential, etc.), which affects the response of mitochondria to the second stimulus. This possibility is ruled out by the experiment presented in Fig. 11, which shows two sequential mitochondrial Ca^{2+} transients in permeabilized cells, similar in amplitude and duration to those evoked by histamine in intact cells. A rapid $[Ca^{2+}]_m$ transient was induced by a short perfusion with a saline solu-

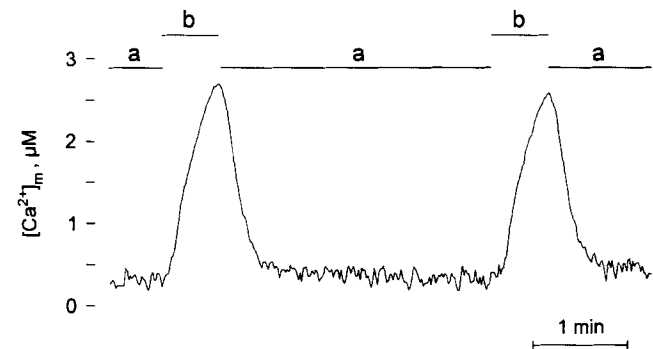


Figure 11. Repetitive $[Ca^{2+}]_m$ transients in permeabilized cells. All conditions as in Fig. 5 *B*. Before recording, the cells were permeabilized by a 2-min incubation with digitonin, added to buffer *a* (see Fig. 5); buffer *b*: as buffer *a*, but the free Ca^{2+} concentration was buffered at $2 \mu M$, as in Fig. 5.

tion containing a buffered Ca^{2+} concentration of $2 \mu\text{M}$, followed by a wash with an EGTA-containing, Ca^{2+} -free, medium. No difference could be observed either in the kinetics or in the amplitude of the two $[\text{Ca}^{2+}]_m$ rises, indicating that the first accumulation of Ca^{2+} within the matrix did not affect the response of mitochondria to a second Ca^{2+} challenge. Further confirmation that the sharp reduction in aequorin light output reflects the existence of mitochondrial subpopulations, is provided by the comparison with similar experiments carried out in a cellular clone expressing nuclear aequorin (Brini et al., 1993). In this case, the light output evoked by successive stimulations was decreased proportionally to aequorin consumption, and thus the $[\text{Ca}^{2+}]_m$ changes, as calculated with a normal calibration procedure, were independent of the order of agonist addition (data not shown).

Discussion

The possibility of monitoring the changes in Ca^{2+} concentration with specifically targeted chimeric aequorins (Rizzuto et al., 1992, 1993; Brini et al., 1993; Rutter et al., 1993) has provided new insight in our understanding of Ca^{2+} dynamics within eukaryotic cells. In this article, we focus our attention on mitochondrial aequorin, with the aim of investigating the role played by mitochondria in the signal transduction pathway initiated by ligand-receptor interaction at the plasma membrane. We also discuss a number of methodological aspects of this new technique. In particular, we here show not only that mitochondrially targeted recombinant aequorin can be efficiently expressed in all tested cells, but also that reconstitution of the functional photoprotein is a simple and nontraumatic procedure: full reconstitution can be obtained by a 2–3-h incubation with the prosthetic group in growth medium, while lower amounts of active photoprotein, sufficient for reliable *in vivo* measurements, can be obtained with shorter incubation times (15–30 min). This simplicity of use extends the use of the methodology to a large variety of cell types, including primary cultures, thereby opening the way for the analysis of Ca^{2+} homeostasis in all the subcellular compartments for which targeting sequences are known and thus suitable aequorin chimeras can be generated. Moreover, the excellent signal to noise ratio, the high efficiency of intracellular targeting and the possibility of monitoring light emission in living cells suggest that chimeric aequorins could also be used as reporter systems for studying biological phenomena not related to Ca^{2+} signaling, such as, for example, protein sorting or gene expression (see, to this regard, the recent review of Miller et al., 1994, on the imaging of aequorin in living cells).

Challenging a variety of cells with compounds which, by acting on plasmalemmal receptors coupled to InsP3 production, induce changes in $[\text{Ca}^{2+}]_c$ produces a large rise in $[\text{Ca}^{2+}]_m$, which exhibits a common pattern: an upstroke phase, with a time course almost indistinguishable from that of $[\text{Ca}^{2+}]_c$, and a fast decay, which rapidly brings $[\text{Ca}^{2+}]_m$ close to resting values. This pattern of response appears to be a general characteristic of the signaling pathway of these receptors, and it could be speculated that the evolutionary success of Ca^{2+} as a second messenger may also be due to

its effect on an organelle, whose activity (energy supply) is fundamental for a stimulated cell.

The process of Ca^{2+} translocation across the inner mitochondrial membrane has been known for over two decades. As to the uptake, we have previously described a large discrepancy between the speed of mitochondrial Ca^{2+} accumulation in permeabilized HeLa cells (which well matches the kinetic properties of the uniporter, as studied in isolated mitochondria) and that in stimulated intact cells. We suggested that mitochondrial Ca^{2+} uptake depends on the microheterogeneity of $[\text{Ca}^{2+}]_c$ changes, i.e., on the generation, upon release of stored Ca^{2+} , of local domains with high Ca^{2+} in the proximity of mitochondria. This notion is further reinforced by the experiments presented here. Mitochondrial Ca^{2+} uptake occurs via the electrophoretic uniporter and is driven by the proton electrochemical gradient, which is built across the inner mitochondrial membrane by the activity of the respiratory complexes. As expected, the best characterized inhibitor of the mitochondrial uniporter, RR, drastically inhibits Ca^{2+} uptake, while the recognized positive effectors, such as spermine, increase the efficiency of mitochondrial Ca^{2+} accumulation.

The high speed which can be detected *in vivo* does not depend on the recruitment of biochemical pathways which were not identified (or lost) in isolated mitochondria or to a direct effect of InsP3 on mitochondrial Ca^{2+} uptake, but rather on the properties of the $[\text{Ca}^{2+}]_c$ dynamics. Indeed, we show that, in intact cells, different mechanisms of Ca^{2+} release from intracellular stores differ markedly in their effects on $[\text{Ca}^{2+}]_m$. In particular, the release of stored Ca^{2+} via a nonspecific leak, such as that induced by blocking the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA), causes an accumulation of Ca^{2+} in the mitochondria which is smaller and slower than that caused by the opening of the InsP3-gated channels, also in experimental conditions in which the $[\text{Ca}^{2+}]_c$ changes are significantly smaller in the latter case. Similarly, in permeabilized cells the effect of SERCA blockers on $[\text{Ca}^{2+}]_m$ is negligible, while perfusion with InsP3 induces a $[\text{Ca}^{2+}]_m$ transient comparable to that observed in intact cells. Altogether, these data indicate that the rapid release from stores and the spatial organization of mitochondria and Ca^{2+} release channels are key elements in the transient generation of those microdomains with high $[\text{Ca}^{2+}]_c$, to which the Ca^{2+} uptake systems of mitochondria are suitably tuned. A similar "coupling" mechanism (high environmental Ca^{2+} + low affinity uptake) protects the organelles against Ca^{2+} overload. Indeed, the local gradients are rapidly dissipated by diffusion, thereby reducing the extramitochondrial $[\text{Ca}^{2+}]_c$ to the average level of the cytoplasm, i.e., values at which the rate of mitochondrial Ca^{2+} accumulation is drastically reduced.

The observation that only a subpopulation of cellular mitochondria is exposed to these microdomains adds further complexity to the system. Indeed, in repeated cell stimulations a reduction can be observed in the light emission evoked by the second agonist, which is not proportional to the amount of aequorin consumed by the first stimulus. It can be excluded that this reduction is attributable to a highly responding cell subpopulation, since the response at the single cell level is quite homogeneous in terms of both amplitude and kinetics. These data therefore suggest the existence in each cell of at least two subpopulations of mitochondria, one

undergoing large $[Ca^{2+}]_m$ changes (and thus consuming a large fraction of its aequorin content) because of their vicinity to the release channels, and the other sensing the mean $[Ca^{2+}]_c$ of the cytosol and thus undergoing much smaller $[Ca^{2+}]_m$ changes. Based on a number of experiments similar to those presented in Fig. 10, the fraction of highly responsive mitochondria can be roughly calculated. In particular, irrespective of the type of agonist, the first challenge consumed 25–30% of all aequorin, the second about 5% of the remaining photoprotein, while the response to the third was negligible. It can therefore be concluded that the first two stimuli consumed virtually all the aequorin of the responding mitochondria, which can thus be estimated to represent about 30–35% of total. This value is sufficiently high to suggest that the apposition of mitochondria close to the $InsP_3$ -gated channel might be not just a stochastic event, but a finely controlled phenomenon.

What is the effect of $[Ca^{2+}]_m$ changes within the mitochondrial matrix? The most appealing hypothesis was first proposed by Denton and McCormack (1980), who demonstrated the Ca^{2+} dependency of three mitochondrial dehydrogenases: the pyruvate dehydrogenase complex, i.e., the entry site of carbon atoms into the Krebs cycle, the NAD^+ -isocitrate dehydrogenase, and the 2-oxoglutarate dehydrogenase complex. These three enzymes catalyze the formation of NADH, which enters the respiratory chain, and are considered potential regulatory sites for the activity of oxidative metabolism. A severalfold enhancement in activity of these enzymes was observed for $[Ca^{2+}]_m$ variations in the low micromolar range (Denton and McCormack, 1980), i.e., the concentrations that we measured in the mitochondria of stimulated cells. Based on these observations, these and other authors proposed that the transfer of the cytosolic Ca^{2+} signal to mitochondria could allow the modulation of the mitochondrial metabolism according to cell needs (Denton and McCormack, 1980, 1985, 1990; McCormack et al., 1990, 1994; Balaban, 1990; Heinemann and Balaban, 1990; Hansford, 1991). Indeed, NADH increases were observed, for example, in sea urchin eggs upon fertilization (Epel, 1964) and in potassium-stimulated adrenal glomerulosa cells (Pralong et al., 1992). Here we provide direct experimental evidence to this general scheme: a marked increase in NADH production can be observed in parallel with the agonist-induced rise in $[Ca^{2+}]_m$. It can be speculated that the use of a common messenger for cytosolic and organelle events represents a simple, effective mechanism for a coordinated "double" signaling. Upon stimulation, Ca^{2+} is released from intracellular stores: the brief local transient is perceived by a significant proportion of mitochondria, thereby increasing their metabolic activity. The Ca^{2+} gradient then diffuses to the rest of the cytoplasm, with two effects: (a) the cytosolic response is elicited, which usually consists of energy-requiring processes (secretion, cell motility, contraction, etc.) and thus is favored by mitochondrial "priming," and (b) mitochondrial Ca^{2+} accumulation quickly ceases, thereby preventing a deleterious organellar Ca^{2+} overload.

Also the rapid return of $[Ca^{2+}]_m$ to close to basal values probably serves the purpose of reducing the futile cycling of Ca^{2+} ions across the inner mitochondrial membrane. Obvious candidates for this role are the mitochondrial Ca^{2+} ex-

changers, which in isolated heart mitochondria have been shown to participate in the control of $[Ca^{2+}]_m$ homeostasis and NADH production (Cox and Matlib, 1993). Based on the effect of specific inhibitors, we now show that the electroneutral Ca^{2+} exchangers are well tuned to the range of the $[Ca^{2+}]_m$ variations which occur in the mitochondrial matrix of living cells and thus actively participate in the control of the $[Ca^{2+}]_m$ transients.

In conclusion, we have shown a direct signaling pathway that spans from the stimulation of a plasma membrane receptor to an internal organelle. It appears that, after the endosymbiotic adaptation of mitochondria, a system has developed for coupling their activity to the cell needs, which is based on the selective uptake of Ca^{2+} ions. In this process, a key role appears to be played by the origin of the $[Ca^{2+}]_c$ changes; in the cells we analyzed here, which are all nonexcitable cells, large $[Ca^{2+}]_m$ changes are evoked by Ca^{2+} release from intracellular stores. In contrast, in excitable cells, where $[Ca^{2+}]_c$ changes mostly depend on the entry of Ca^{2+} from the extracellular medium, $[Ca^{2+}]_m$ rises are mostly evoked by the opening of plasmalemmal channels (Rutter et al., 1993; Rizzuto, R., manuscript in preparation). It appears that the way for activating mitochondria depends on the type of stimulation affecting the cell, and that the intracellular distribution of the organelles plays a key role in modulating this process.

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