

# Beat-to-beat oscillations of mitochondrial $[Ca^{2+}]_m$ in cardiac cells

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**The  $Ca^{2+}$ -sensitive photoprotein aequorin and the new green fluorescent protein-based fluorescent  $Ca^{2+}$  indicators 'ratiometric-pericam' were selectively expressed in the mitochondria, cytosol and/or nucleus of spontaneously beating ventricular myocytes from neonatal rats. This combined strategy reveals that mitochondrial  $[Ca^{2+}]_m$  oscillates rapidly and in synchrony with cytosolic and nuclear  $[Ca^{2+}]_c$ . The  $Ca^{2+}$  oscillations were reduced in frequency and/or amplitude by verapamil and carbachol and were enhanced by isoproterenol and elevation of extracellular  $[Ca^{2+}]_c$ . An increased frequency and/or amplitude of cytosolic  $Ca^{2+}$  spikes was rapidly mirrored by similar changes in mitochondrial  $Ca^{2+}$  spikes and more slowly by elevations of the interspike  $Ca^{2+}$  levels. The present data unequivocally demonstrate that in cardiac cells mitochondrial  $[Ca^{2+}]_m$  oscillates synchronously with cytosolic  $[Ca^{2+}]_c$  and that mitochondrial  $Ca^{2+}$  handling rapidly adapts to inotropic or chronotropic inputs.**

**Keywords:** aequorin/contraction/mitochondrial  $Ca^{2+}$ /neonatal cardiac cells/pericam

## Introduction

Although mitochondrial  $Ca^{2+}$  transport was discovered >30 years ago, only in recent years have the various aspects of this system and its importance in cell physiology become generally accepted (Duchen, 1999). In cardiac cells, however, the role of mitochondrial  $Ca^{2+}$  handling is still debated; in particular, its capacity to follow the beat-to-beat changes of cytosolic calcium concentration ( $[Ca^{2+}]_c$ ). Two major factors have contributed to the controversy. First, given the low affinity of the mitochondrial  $Ca^{2+}$  uptake mechanism, it was difficult to envisage how these organelles could respond adequately to the relatively small amplitude of  $[Ca^{2+}]_c$  changes observed under normal physiological conditions (Fry *et al.*, 1984). This objection has been largely overcome by the demonstration that in many cells, including cardiomyocytes, the strategic location of mitochondria close to the  $Ca^{2+}$  release channels (and therefore to micro-

domains of very high  $[Ca^{2+}]_c$ ), permits substantial mitochondrial  $Ca^{2+}$  uptake, despite modest average changes in  $[Ca^{2+}]_c$  (Rizzuto *et al.*, 1993; Robert *et al.*, 2001). In agreement with this, Ramesh *et al.* (1998) showed that in adult cardiac cells the  $Ca^{2+}$  release induced by caffeine generates micro-domains of elevated  $[Ca^{2+}]_c$  that can be high enough to be sensed by neighboring mitochondria even in the presence of high doses of a cytosolic  $Ca^{2+}$  buffer. Secondly, kinetic measurements obtained in isolated mitochondria suggested that the uniport/antiport system was too slow to respond in the 150–200 ms range that is typical of  $[Ca^{2+}]_c$  changes (Gunter *et al.*, 1998). In addition, the newly discovered RaM system (rapid mode of uptake) required >60 s to recover after a  $Ca^{2+}$  pulse, thus rendering this mechanism inadequate for a rapid and repetitive transmission of cytosolic  $Ca^{2+}$  variations into mitochondria (Buntinas *et al.*, 2001). In intact cardiac cells, measurements of mitochondrial calcium concentrations ( $[Ca^{2+}]_m$ ), have led to conflicting results as to the possible beat-to-beat variations in  $[Ca^{2+}]_m$ , depending on the techniques and species used (Griffiths, 1999; for a recent review see Huser *et al.*, 2000). Several groups showed that  $[Ca^{2+}]_m$  can be monotonically raised to 600–750 nM with combined  $\beta$ -adrenergic and electrical stimulations, but no repetitive oscillations were observed under these conditions (Miyata *et al.*, 1991; Di Lisa *et al.*, 1993; Griffiths *et al.*, 1997). On the contrary, Isenberg *et al.* (1993) were the first to provide evidence for oscillatory changes in total mitochondrial calcium during the contraction–relaxation cycle using electron probe microanalysis. Their conclusions were later supported by experiments performed with fluorescent indicators (Chacon *et al.*, 1996; Trollinger *et al.*, 2000). Thus, while it is now largely accepted that mitochondrial  $Ca^{2+}$  transport can be activated in cardiac cells when either the sarcoplasmic reticulum  $Ca^{2+}$  release is pharmacologically stimulated or  $[Ca^{2+}]_c$  is continuously elevated above a threshold level, it still remains highly controversial whether  $[Ca^{2+}]_m$  can respond to the rapid  $[Ca^{2+}]_c$  changes occurring during each cycle of contraction (Huser *et al.*, 2000). The major obstacle to reaching a definitive conclusion depends on the methodologies that have been employed to measure  $[Ca^{2+}]_m$ . In the studies mentioned above, fluorescent  $Ca^{2+}$  indicators have been loaded into cells and within mitochondria via their membrane-permeable AM esters. These dyes accumulate in mitochondria to different extents: indo-1 = fura-2 < rhod-2. Invariably these probes are also present in the cytosol and several approaches have been used to selectively remove them from the cytosolic compartment. In addition, the dyes accumulate in organelles other than mitochondria, hampering the interpretation of the fluorescence changes. These fluorescent dyes also present other problems, such as (i) their  $Ca^{2+}$ -buffering capacity, (ii) possible effects of

the AM hydrolysis products on mitochondrial physiology and (iii) movement artifacts (Takahashi *et al.*, 1999).

Increases of [Ca<sup>2+</sup>]<sub>m</sub> are pivotal in the activation of mitochondrial dehydrogenases and thus are essential for adjusting ATP production to cardiac needs during contraction (Hansford *et al.*, 1998; Jouaville *et al.*, 1999). On the other hand, given that [Ca<sup>2+</sup>]<sub>c</sub> oscillates on a beat-to-beat basis, it remains unclear how this cytosolic information is transmitted to mitochondria. In other words, are the rapid cytosolic Ca<sup>2+</sup> changes transmitted as slow tonic increases or as synchronous oscillations of [Ca<sup>2+</sup>]<sub>m</sub>?

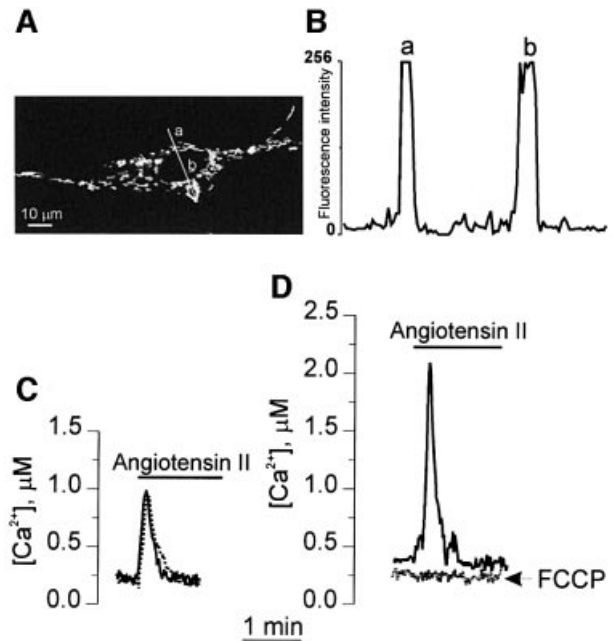
In the present work we have sought to address two questions. First, do beat-to-beat [Ca<sup>2+</sup>]<sub>m</sub> changes occur? And, secondly, if so, are these capable of adapting to physiological changes in [Ca<sup>2+</sup>]<sub>c</sub> imposed by chronotropic and inotropic stimuli?

To this end, we adopted a novel methodological approach, combining genetically encoded targeted Ca<sup>2+</sup> probes, aequorins and pericams, with spontaneously contracting neonatal cardiomyocytes in culture. The results obtained with this system demonstrate that: (i) mitochondria display repetitive oscillations in their [Ca<sup>2+</sup>]<sub>m</sub>; (ii) these oscillations are synchronous with [Ca<sup>2+</sup>]<sub>c</sub> changes; and (iii) the [Ca<sup>2+</sup>]<sub>m</sub> oscillations are sensitive to modulations of the amplitude and frequency of [Ca<sup>2+</sup>]<sub>c</sub> changes.

## Results

### Expression and subcellular distribution of recombinant aequorins in neonatal cardiac cells

A prerequisite of this study was finding a protocol that allowed the expression of aequorin chimeras at sufficiently high levels to obtain an acceptable signal-to-noise ratio in neonatal cardiac cells. Several classical transfection protocols (calcium phosphate, electroporation, liposomes, etc.) have been tested with unsatisfactory results. This difficulty was overcome by the use of FuGENE 6, a lipid-based, non-liposomal transfection reagent. With this procedure, we obtained ~10% of transfected cells, the morphology and spontaneous contractions of which were indistinguishable from those of their non-transfected counterparts. We employed two aequorin constructs: the first, cyt-Aeq, localizes to the cytoplasm, whereas the other, mit-Aeq, is targeted specifically to the mitochondrial matrix (Rizzuto *et al.*, 1993, 1996). The distribution of mitochondrial aequorin and of the untargeted aequorin was tested by immunocytochemistry with specific antibodies (data not shown) and also by transfecting the cells with constructs where the aequorin sequence was substituted by that encoding green fluorescent protein (GFP). We have previously shown that the subcellular localization of mit-GFP and mit-Aeq is identical (Rizzuto *et al.*, 1996). As for all other cell types tested thus far, cytosolic GFP or aequorin were diffusely distributed in the cytosol and the nucleus (not shown). Figure 1A shows that cardiomyocytes transfected with the mitochondrial-targeted GFP expressed the fluorescent protein exclusively within elongated organelles with the characteristic morphology of mitochondria. Quantitative analyses of the confocal image showed that the fluorescent signal in regions corresponding to the cytosol or nucleus was indistinguishable from background (Figure 1B). The



**Fig. 1.** Subcellular distribution of mitochondria-targeted aequorin. (A) Image of a cardiomyocyte expressing mit-GFP obtained by confocal microscopy (Rizzuto *et al.*, 1996). The subcellular distribution of mit-GFP is indistinguishable from that of mit-Aeq. The profile of the fluorescent intensity along the line depicted in (A) is presented in (B); a and b are regions corresponding to the mitochondria as presented in (A). The level of fluorescence in regions of the cytoplasm devoid of mitochondria and in the nuclear region was indistinguishable from the level of fluorescence in non-transfected cells and was at least 50 (or more)-fold lower than in regions rich in mitochondria. The kinetics of [Ca<sup>2+</sup>]<sub>c</sub> (C) and [Ca<sup>2+</sup>]<sub>m</sub> (D) changes following stimulation with 1 µM angiotensin II are presented, in the presence (dotted trace) and absence (continuous trace) of 4 µM FCCP. In this experiment the cells were plated at low density to avoid the formation of a syncytium, the signal was integrated for 1 s and calibrated in terms of [Ca<sup>2+</sup>] as described by Brini *et al.* (1995). Under these conditions the oscillatory pattern of [Ca<sup>2+</sup>] is not evident.

subcellular localization of mit-Aeq was further confirmed by biochemical and functional experiments. Treatment of cardiac cells transfected with mit-Aeq with 50 µM digitonin caused the release in the supernatant of most of the cytosolic marker enzyme, lactic dehydrogenase and trace amounts of both the mitochondrial enzyme marker, citrate synthase and aequorin. Subsequent treatment with Triton X-100 released in the second supernatant equal percentages of the mitochondrial marker citrate synthase and of the total aequorin content (Table I). Functional evidence for the localization of aequorin exclusively in the mitochondrial matrix was then explored (Figure 1). Cells expressing mitochondrial- or cytosolic-targeted aequorin were stimulated with angiotensin II. The peptide triggered a rapid and transient [Ca<sup>2+</sup>] increase in both compartments (Figure 1C and D). In the presence of the mitochondrial uncoupler FCCP the increase of [Ca<sup>2+</sup>]<sub>m</sub> was blocked while that of [Ca<sup>2+</sup>]<sub>c</sub> was unaffected. Based on these data, it can be concluded that the percentage of mitochondrial aequorin mistargeted to the cytosol is undetectable.

### Reconstitution efficiency of targeted aequorin

In order to obtain functional aequorin, the expressed apoprotein moiety must be reconstituted with its co-

**Table I.** Release of marker enzymes and mitochondrial aequorin by digitonin and Triton X-100

|                       | Supernatant<br>after digitonin<br>(% of total) | Supernatant<br>after Triton<br>(% of total) |
|-----------------------|--|---|
| Lactate dehydrogenase | 91.6 ± 1.1                                     | 8.4 ± 0.8                                   |
| Citrate synthase      | 4.9 ± 0.4                                      | 95.1 ± 2.1                                  |
| Aequorin              | 4 ± 1  | 96 ± 2                                      |

The experimental conditions are described in Materials and methods. Values are expressed as percentage of enzymatic activity and aequorin content ( $n = 3$ ).

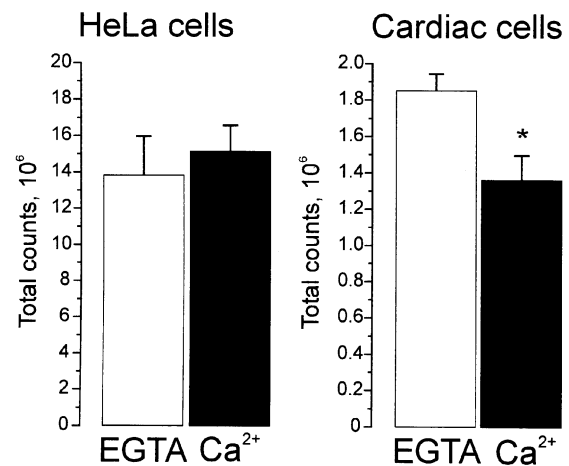
enzyme, coelenterazine; this is added to the medium and freely diffuses into the cell. The efficiency of reconstitution depends not only on the incubation time and coelenterazine concentration, but also on the level of  $\text{Ca}^{2+}$  in the compartment where aequorin is localized (Robert *et al.*, 2000). In all cell types tested thus far, reconstitution of the apoprotein in the cytosol and mitochondria is highly efficient in saline solutions or media containing physiological  $[\text{Ca}^{2+}]_i$ ; this is as expected, given the very low (100–200 nM) and stable levels of  $\text{Ca}^{2+}$  in these compartments under unstimulated conditions (Rizzuto *et al.*, 1993). The situation in cardiomyocytes, however, is different. These cells undergo continuous spontaneous contractions, driven by transient increases in  $[\text{Ca}^{2+}]_i$ . In order to verify whether the efficiency of reconstitution could provide indirect information on the level of  $\text{Ca}^{2+}$  in the cytosol, two protocols of reconstitution with coelenterazine were compared; in one, a saline solution containing 1 mM  $\text{CaCl}_2$  was used, whereas in the other the  $\text{CaCl}_2$  was omitted and 100  $\mu\text{M}$  EGTA was used instead. The two protocols were applied to two different cell types, HeLa cells and cardiomyocytes, i.e. cells whose resting  $[\text{Ca}^{2+}]_i$  is stable (HeLa) or oscillates (cardiomyocytes). Figure 2A shows the results obtained with cyt-Aeq. The total number of luminescence counts (reflecting the efficiency of reconstitution) was practically identical in HeLa cells, independent of the  $[\text{Ca}^{2+}]_i$  in the medium. In the case of cardiomyocytes, these levels were significantly different; ~30% lower in media containing 1 mM  $\text{CaCl}_2$ .

The question then arises as to whether cells expressing mit-Aeq are subjected to a similar phenomenon. Figure 2B shows that the efficiency of reconstitution of mit-Aeq in HeLa cells was unaffected by the presence of extracellular  $\text{Ca}^{2+}$ . In cardiac myocytes however, the total amount of luminescence obtainable was again reduced (this time by ~45%) when cells were reconstituted in the presence of  $\text{Ca}^{2+}$  as compared with parallel batches of cells that were reconstituted in  $\text{Ca}^{2+}$ -free solution.

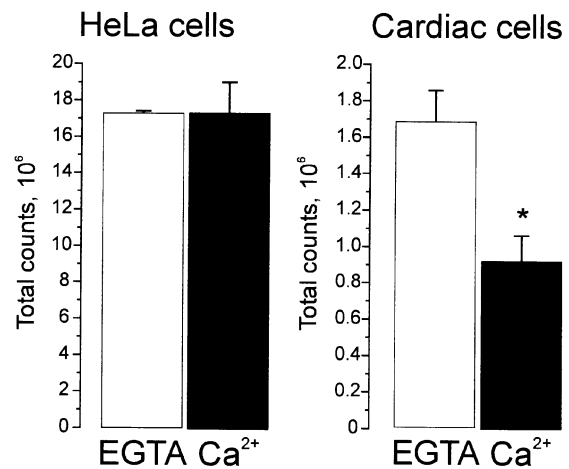
### Spontaneous repetitive elevations of $[\text{Ca}^{2+}]_i$ and $[\text{Ca}^{2+}]_m$ in beating cardiac cells

The reduced efficacy of reconstitution in contracting cells may reflect either a higher steady state level or beat-to-beat changes in  $[\text{Ca}^{2+}]_m$ . The direct approach to the problem would be to measure  $[\text{Ca}^{2+}]_m$  in single cells. This is technically very demanding and the level of recombinant photoprotein that can be obtained in cardiomyocytes is too low for this technique to be applicable with an adequate

## A Cytosol

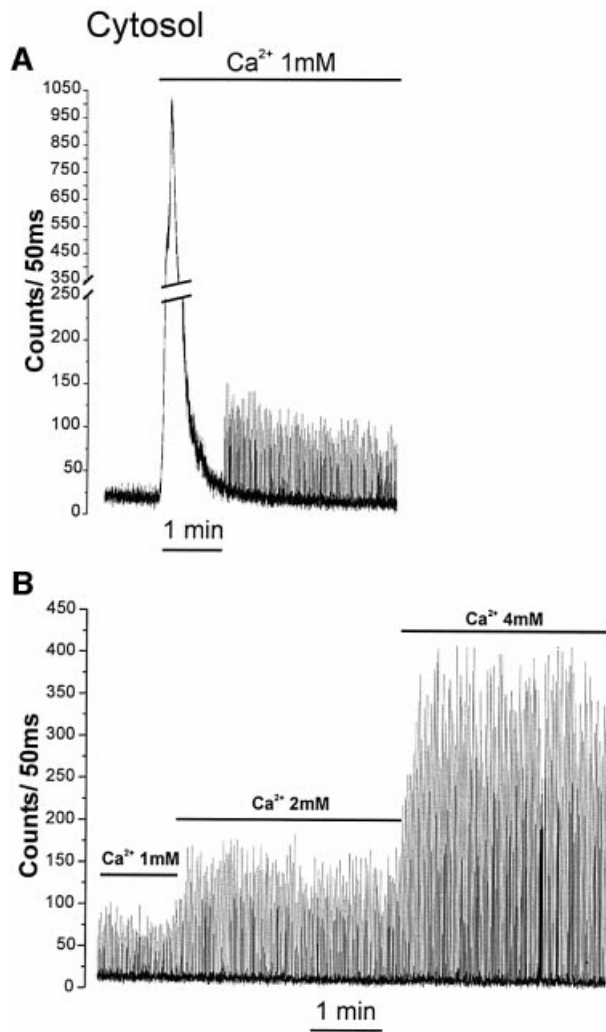


## B Mitochondria



**Fig. 2.** Reconstitution efficiency of targeted aequorins. HeLa cells and cardiomyocytes were transfected with chimeras that encode aequorin targeted either to the cytosol (A) or to mitochondria (B). Reconstitution of the functional aequorin was carried out, as described in detail in Materials and methods, by incubating the cells with coelenterazine in saline medium containing either  $\text{CaCl}_2$  or EGTA. After 1 h of incubation, the total light output was measured by lysing the cells with digitonin, in the presence of 10 mM  $\text{CaCl}_2$ . \* $p < 0.05$ . Values are mean  $\pm$  SD ( $n = 8$ ).

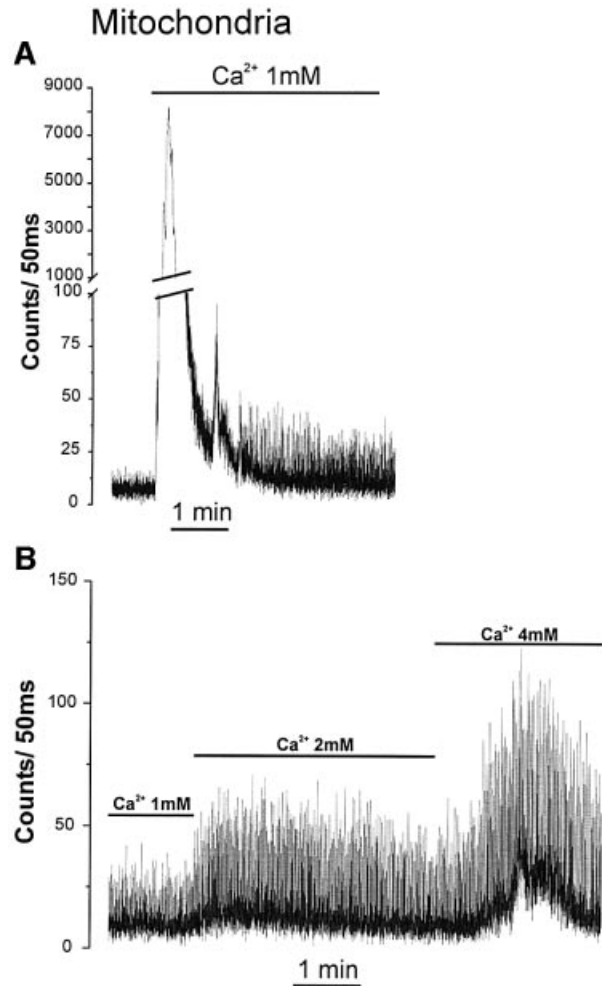
time resolution. To bypass this limitation, we took advantage of the fact that when cardiomyocytes are plated at confluence, the vast majority of cells contract synchronously (Kucera *et al.*, 2000). Under these conditions, the cells behave as a syncytium and the average signal measured over the entire cell population closely reflects the behavior of each single cell. To confirm the validity of this system, we used cyt-Aeq to measure the well described  $\text{Ca}^{2+}$  oscillations in contracting cardiomyocytes. After the step of reconstitution with coelenterazine under  $\text{Ca}^{2+}$ -free conditions, the light output of the whole population was analyzed without added stimuli; the light output was fairly constant, with a background noise of ~10 counts/50 ms (Figure 3A). Addition of 1 mM  $\text{CaCl}_2$  resulted in a transient increase in photon emission. Following this initial peak, the average luminescence signal was characterized by rapid regular spikes, the



**Fig. 3.** Effect of extracellular  $[\text{Ca}^{2+}]$  on cytosolic  $\text{Ca}^{2+}$  oscillations. Cells were transfected with cyt-Aeq. Reconstitution with coelenterazine was carried out in  $\text{Ca}^{2+}$ -free medium. Where indicated, 1, 2 or 4 mM  $\text{CaCl}_2$  was added to the perfusion medium. On the ordinate, the aequorin luminescence (counts/50 ms) is presented.

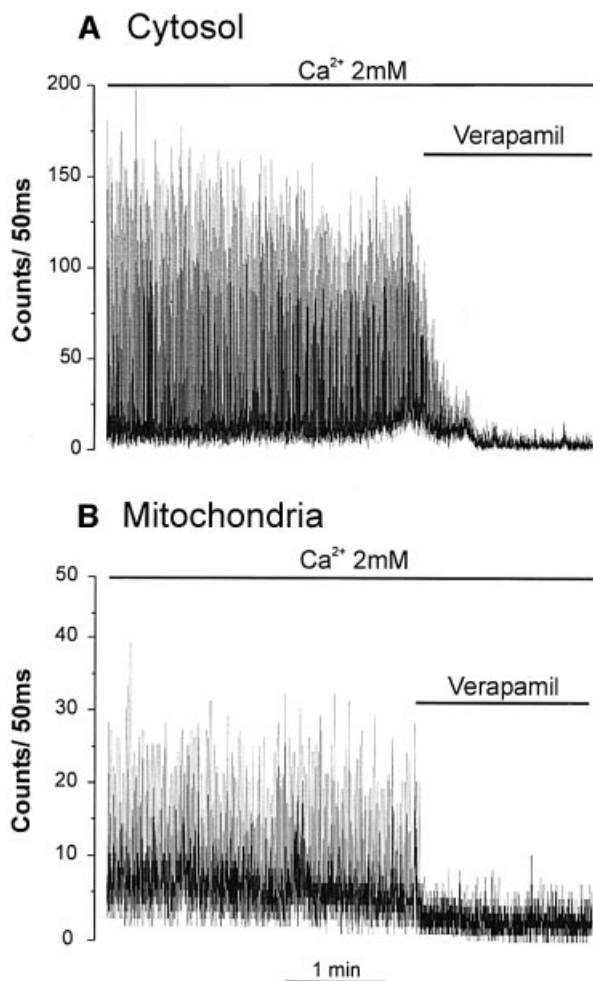
amplitude and frequency of which varied slightly in different experiments. Figure 3B shows that the amplitude of these spikes increased if the extracellular  $[\text{Ca}^{2+}]$  was raised from 1 to 2 or 4 mM. The kinetics of cytoplasmic  $\text{Ca}^{2+}$  changes were monitored in parallel batches of cells loaded with fura-2 and found to match the results obtained with cyt-Aeq (data not shown).

A similar oscillatory behavior was displayed by cells expressing mit-Aeq. Addition of 1 mM  $\text{CaCl}_2$  resulted in a large luminescence peak, followed by spontaneous spikes (Figure 4A). Progressive elevation of extracellular  $[\text{Ca}^{2+}]$  increased the amplitude of the  $\text{Ca}^{2+}$  spikes also in the mitochondria (Figure 4B). One aspect differentiates this pattern from that observed in the cytosol: there was an increased interspike luminescence level with increasing extracellular  $[\text{Ca}^{2+}]$ ; this was particularly evident when cells were perfused with 4 mM  $\text{CaCl}_2$ . Both cytosolic and mitochondrial  $\text{Ca}^{2+}$  spikes were abolished by 20  $\mu\text{M}$  verapamil (Figure 5). Representative traces are shown with an expanded time scale in Figure 6. In the case of cytosolic luminescence spikes, the peak was reached in



**Fig. 4.** Effect of extracellular  $[\text{Ca}^{2+}]$  on mitochondrial  $\text{Ca}^{2+}$  oscillations. Cells were transfected with mit-Aeq. Other conditions are as in Figure 3.

~50–100 ms, while the decay phase took 100–150 ms. Given the time resolution of our measuring apparatus (1 data point every 50 ms) these values are only approximate. The mitochondrial spikes show somewhat slower kinetics; ~100–150 ms to reach the peak and 200–300 ms to return close to basal levels. Increasing the extracellular  $[\text{Ca}^{2+}]$  only marginally modified the kinetics of  $[\text{Ca}^{2+}]_m$  rise, but the decay time at high extracellular  $[\text{Ca}^{2+}]$  showed a clear increase, thus accounting for the ‘diastolic’ increase in  $[\text{Ca}^{2+}]_m$  observed at 4 mM  $\text{CaCl}_2$ . With the present system, it is difficult to correctly correlate luminescence and absolute  $\text{Ca}^{2+}$  concentrations. This is primarily due to the impossibility of determining the exact proportion of transfected cells that contract synchronously in individual experiments. If, however, one assumes that all transfected cells contract synchronously, a lower limit can be calculated. Given this assumption, we determined that at 2 mM  $\text{CaCl}_2$  in the medium the cytosolic and mitochondrial  $\text{Ca}^{2+}$  spikes reach values of  $0.72 \pm 0.09 \mu\text{M}$  and  $0.36 \pm 0.03 \mu\text{M}$  ( $n = 8$ ), respectively. This latter value may be a further underestimate of the true  $[\text{Ca}^{2+}]_m$  peaks if, as reported in other cells, a microheterogeneity exists among mitochondria of individual cells (Montero *et al.*, 2000).

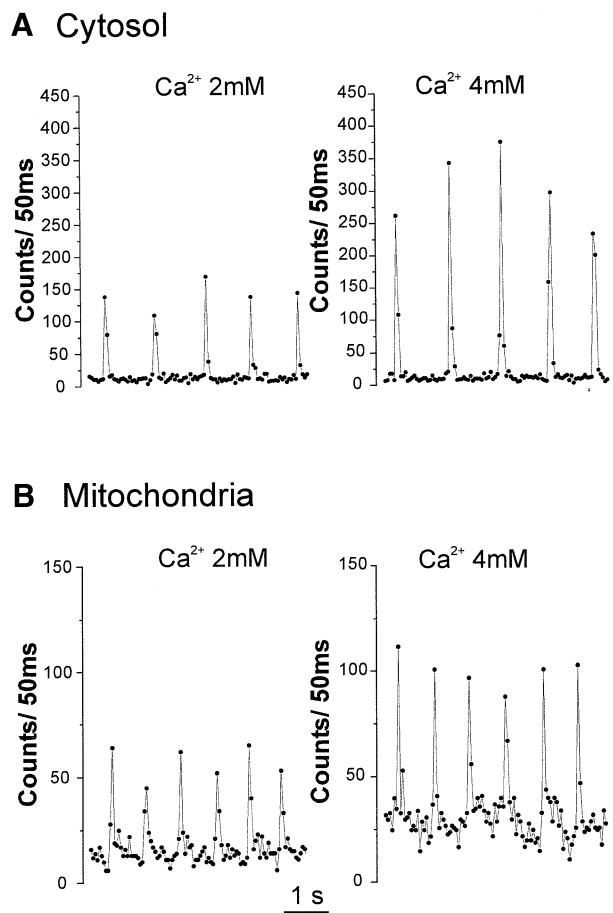


**Fig. 5.** Effect of verapamil on cytosolic (A) and mitochondrial (B)  $\text{Ca}^{2+}$  oscillations. The perfusion buffer contained 2 mM  $\text{CaCl}_2$ . Where indicated, 20  $\mu\text{M}$  verapamil was added.

Inhibitors of the uniporter such as Ruthenium Red or Ru360 were totally inefficient in intact cells. Upon permeabilization of the plasma membrane with digitonin, however, both drugs completely blocked mitochondrial  $\text{Ca}^{2+}$  uptake. This implies that, unlike their adult counterparts, neonatal cardiomyocytes are impermeant to these drugs (Matlib *et al.*, 1998). Uncouplers in the absence or presence of oligomycin resulted in a complete block not only of mitochondrial spiking but, after a delay of a few seconds, also of the cytoplasmic oscillations (data not shown).

#### **Effects of inotropic and chronotropic stimuli on $[\text{Ca}^{2+}]_m$**

One major question is whether mitochondrial  $\text{Ca}^{2+}$  spikes could be modulated by physiological stimuli that modify the amplitude or the frequency of the contraction-relaxation cycles. To this end, we first recorded the effect of isoproterenol, a  $\beta$ -adrenergic agonist that displays positive inotropic and chronotropic effects. Cells transfected either with cyt-Aeq or mit-Aeq (and perfused with 2 mM  $\text{CaCl}_2$ ), showed an augmentation of both spike amplitude and frequency when treated with isoproterenol (Figure 7). In mitochondria, the increase in frequency was

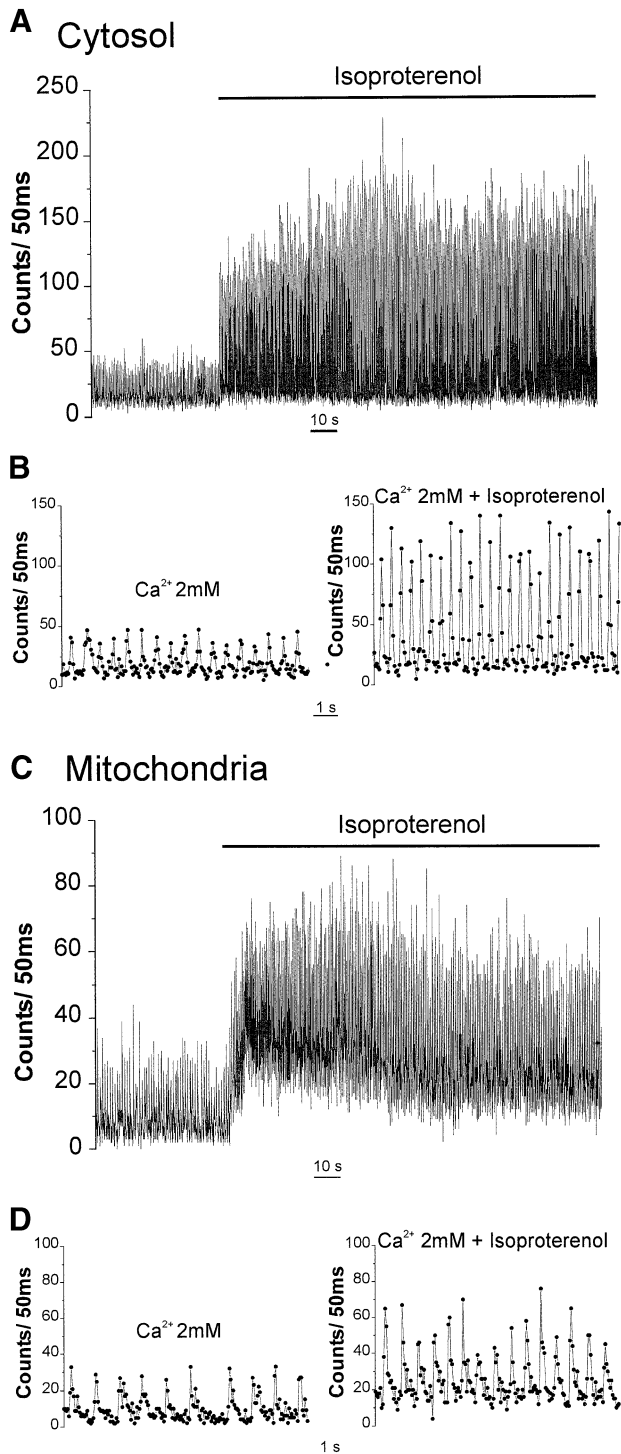


**Fig. 6.** Fast kinetic analysis of cytosolic (A) and mitochondrial (B)  $\text{Ca}^{2+}$  oscillations. The data are details of the kinetics shown in Figure 4 and are presented on an expanded time scale.

always accompanied by an increase in the interspike level. Statistical analyses of different cell preparations showed that the average frequency of the spikes in unstimulated conditions was  $\sim 1$  Hz for both compartments; the increase in frequency induced by isoproterenol ( $\sim 50\%$ ) was again indistinguishable in the two compartments (Figure 8). As for the changes in spike amplitude caused by isoproterenol, the average increase observed in the cytosol was  $>2$ -fold, whereas in mitochondria a more modest increase ( $\sim 70\%$ ) was seen (data not shown). The effect of carbachol, a muscarinic agonist that evokes negative chronotropic and inotropic effects, was then explored. Figure 9 shows that carbachol reduced the amplitude and frequency of both cytoplasmic and mitochondrial  $\text{Ca}^{2+}$  oscillations.

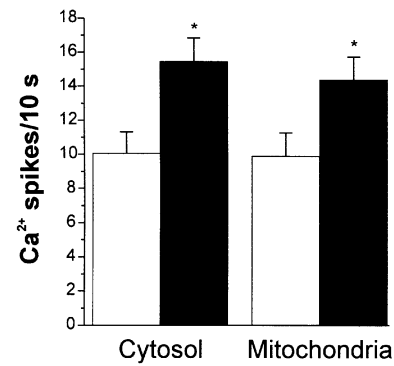
#### **Cytoplasmic and mitochondrial $\text{Ca}^{2+}$ oscillations in single cells**

The major limitation of aequorin in our conditions is the necessity of comparing mitochondrial and cytosolic  $\text{Ca}^{2+}$  oscillations in parallel batches of cells. To overcome this problem, we took advantage of a new family of GFP-derived  $\text{Ca}^{2+}$  indicators called pericams, recently described by Nagai *et al.* (2001). These probes are genetically encoded and can be targeted with high selectivity to different compartments, including the mitochondrial matrix. One of the pericams, called 'ratiometric-pericam'

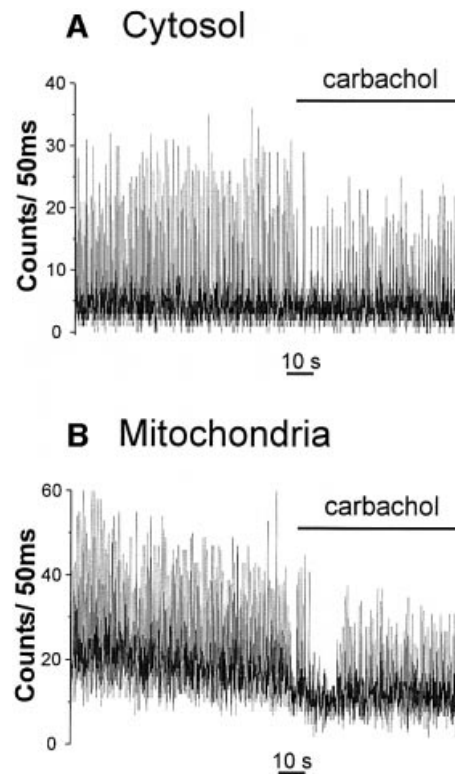


**Fig. 7.** Effect of isoproterenol on cytosolic (A and B) and mitochondrial (C and D) Ca<sup>2+</sup> oscillations. In (B) and (D) part of the kinetics of (A) and (C), respectively, are presented on an expanded time scale. Where indicated, 1  $\mu$ M isoproterenol was added. The medium contained 2 mM CaCl<sub>2</sub>.

changes its excitation spectrum upon Ca<sup>2+</sup> binding and can be used as a 'ratiometric' probe. Correct localization of the mitochondrial Ca<sup>2+</sup> probe was verified by morphometric analysis (Figure 10A). The frequency of mitochondrial Ca<sup>2+</sup> oscillations (on average 1 Hz), as measured in single cells, was indistinguishable from that measured with aequorin, although the decay of the [Ca<sup>2+</sup>]<sub>m</sub> spike toward

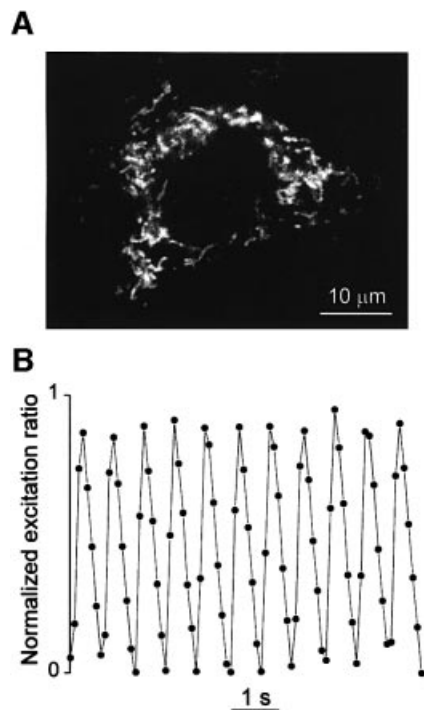


**Fig. 8.** Average frequency of cytosolic and mitochondrial Ca<sup>2+</sup> spikes before (white column) and after (black column) isoproterenol (1  $\mu$ M) addition. Values are mean  $\pm$  S.D. ( $n = 8$ ). \* $p < 0.05$ .



**Fig. 9.** Effect of 500 nM carbachol on cytosolic (A) and mitochondrial (B) Ca<sup>2+</sup> oscillations. The medium contained 2 mM CaCl<sub>2</sub>. Other conditions are as in Figures 3 and 4.

basal level was less sharp than that revealed by aequorin (Figure 1B). To directly prove that the oscillations of cytosolic and mitochondrial [Ca<sup>2+</sup>] are synchronous, cells were cotransfected with two ratiometric-pericams, one targeted to the mitochondria and the other to the nucleus. A nuclear pericam was preferred to a cytoplasmic one because the signals coming from the two compartments can be monitored simultaneously and are not spatially overlapped. It has been previously shown in several cell types that the kinetic delay between nucleoplasmic and cytoplasmic [Ca<sup>2+</sup>] changes is very short (Lipp *et al.*, 1997) and we have confirmed this observation in cardiomyocytes using fura-2 (not shown). The [Ca<sup>2+</sup>] of both compartments oscillated in close synchronicity. The effect of verapamil,



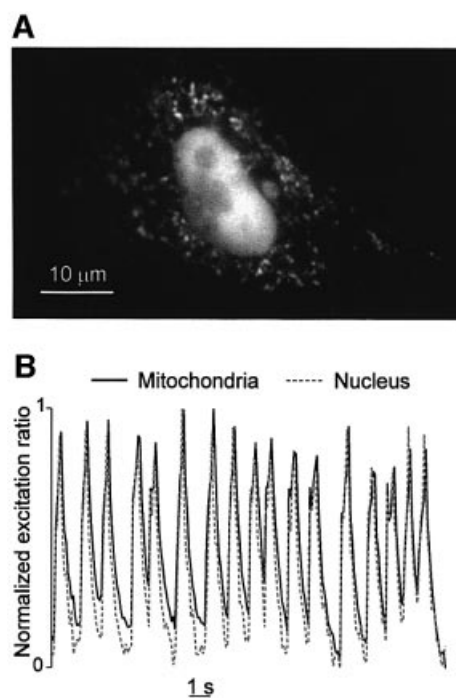
**Fig. 10.** Spontaneous  $[Ca^{2+}]$  oscillations measured with 'ratiometric-pericams' targeted to the mitochondria. Typical image of a neonatal cardiac cell expressing the ratiometric-pericam targeted to the mitochondria (A). The cell was illuminated at 415 nm for 200 ms. (B) The cells were illuminated in sequence for 50 and 20 ms at 415 and 490 nm. The emitted light (at 510 nm) was collected and the ratio between the intensities of the two images (415/490) was calculated off line. On the left side the normalized fluorescence emission ratio is presented. Normalization was carried out by setting the minimum value to 0 and the maximum peak of the 415/490 ratio to 1.

EGTA, carbachol and isoproterenol was also studied in cells expressing the pericams and the results were identical to those obtained with aequorin.

## Discussion

The question of whether the regulation of mitochondrial  $Ca^{2+}$ -dependent dehydrogenases depends on tonic, low amplitude  $[Ca^{2+}]_m$  increases or on frequency modulation of  $[Ca^{2+}]_m$  spiking may have profound consequences on our understanding of the control of energy balance in physiology and possible alterations in pathology.

Given that the conflicts between models are to a large extent due to the limitations of the techniques used so far, we approached the question from a different experimental angle. To this end, we took advantage of two genetically encoded  $Ca^{2+}$  probes, aequorins and ratiometric-pericams, targeted to defined cellular compartments (Pozzan *et al.*, 1994; Nagai *et al.*, 2001). Targeted aequorin and pericam offer two main advantages compared with the classical  $Ca^{2+}$  indicators employed in this field: (i) they are selectively localized (the totality of the probe is confined to the mitochondrial matrix; in the case of fluorescent probes used in previous studies only a proportion of the dye labels this organelle); (ii) they minimize movement artifacts. However, the aequorin system presents one major difficulty, i.e. measuring  $[Ca^{2+}]$  changes at the



**Fig. 11.** Spontaneous  $[Ca^{2+}]$  oscillations measured with 'ratiometric-pericams' targeted to the nucleus and the mitochondria. (A) Typical image of neonatal cardiac cells expressing both the nuclear and mitochondrial ratiometric-pericam. The cells were illuminated at 415 nm for 200 ms. (B) Time course of the 415/490 (normalized) ratio changes in both compartments. The kinetics of a region of interest taken over mitochondria or nucleus are presented as continuous or dotted lines, respectively (B). Other conditions as in Figure 10B.

single cell level with high temporal resolution. This was overcome by the use of neonatal cardiomyocytes plated at confluence. In this condition the cells form synchronously contracting cell monolayers, driven by a focal pacemaker (Kucera *et al.*, 2000). In practical terms, this system allows a large number of cells in culture to be treated as a single spontaneously contracting cell. In addition, it also avoids the necessity for external stimulation or the use of a stimulating electrode. The validity of our experimental model was verified by the use of an aequorin targeted to the cytosol, where the kinetics of  $[Ca^{2+}]_c$  changes are well established. The results obtained with aequorin in a cardiac cell population were complemented by those obtained at the single cell level with the recently described GFP-based  $Ca^{2+}$  probe 'ratiometric-pericam'. This new genetically encoded probe is strongly fluorescent, allowing imaging of  $[Ca^{2+}]$  changes at the single cell level. The availability of ratiometric-pericams allowed us to determine in a more direct way and in the same cells, the synchronicity between nucleoplasmic, and thus cytoplasmic oscillations, based on the observation that there is a very short delay between changes in cytoplasmic and nuclear  $[Ca^{2+}]$  and mitochondrial  $[Ca^{2+}]$  oscillations. Taken together, the experiments carried out with aequorin and ratiometric-pericam-expressing cells demonstrate that  $[Ca^{2+}]_m$  oscillates in parallel with  $[Ca^{2+}]_c$ .

The observation that mitochondrial  $Ca^{2+}$  oscillations are modulated in frequency and amplitude by  $\beta$ -adrenergic and muscarinic stimulations is of major relevance. It demonstrates that mitochondrial  $Ca^{2+}$  transport is responsive to the most important stimuli that control inotropic

and chronotropic inputs. In addition to the possibility of controlling mitochondrial Ca<sup>2+</sup> spikes both in amplitude and frequency, this system shows a third level of control: increase of mitochondrial 'diastolic' [Ca<sup>2+</sup>]. This occurs when the extracellular [Ca<sup>2+</sup>] is increased, or upon isoproterenol stimulation. Cytosolic diastolic Ca<sup>2+</sup> levels, on the other hand, are only marginally affected. This discrepancy between cytosolic and mitochondrial responses may be explained by the relatively slow rate of the mitochondrial efflux pathway; when the spike amplitude and/or frequency is increased this results in an incomplete Ca<sup>2+</sup> extrusion from mitochondria during each spike and the consequent diastolic [Ca<sup>2+</sup>]<sub>m</sub> increase.

The key physiological question is the advantage conferred by such a sophisticated coupling of mitochondrial and cytosolic Ca<sup>2+</sup>. At the moment we can only speculate. The data presented show that cardiac cells possess three modes of Ca<sup>2+</sup> communication between the cytosol and mitochondria; variations in both frequency and peak amplitude of Ca<sup>2+</sup> spikes, as well as [Ca<sup>2+</sup>]<sub>m</sub> diastolic levels. The system is thus highly versatile, enabling these cells to respond accurately to different demands.

Frequency-encoding of the Ca<sup>2+</sup> signal is often the preferred language of both excitable and non-excitable cells and many Ca<sup>2+</sup>-sensitive targets are designed to decode signals that vary both in frequency and in amplitude (Hajnoczky *et al.*, 1995; Dolmetsch *et al.*, 1998). For cardiac cells, the decoding of an increased frequency of cytosolic Ca<sup>2+</sup> oscillations is much faster (practically within one beat) than a change in steady state amplitude (which would require several beats). Indeed the diastolic mitochondrial [Ca<sup>2+</sup>] also appears to adapt (but more slowly), to larger amplitude cytosolic Ca<sup>2+</sup> spikings. In other words, the energy requirements imposed by a rapid and transient increase in chronotropism could hardly be coupled to an increased activity of the intramitochondrial dehydrogenases if [Ca<sup>2+</sup>]<sub>m</sub> were to vary slowly, that is, tonically, to achieve a new steady state. In addition, it can not be excluded that the local uptake by strategically located mitochondria of the Ca<sup>2+</sup> released from the sarcoplasmic reticulum (or entering through Ca<sup>2+</sup> channels) might be relevant for the feedback regulation by Ca<sup>2+</sup> of the channels themselves; a similar scenario has been suggested for InsP3 receptors or CRAC channels in other model systems (Hoth *et al.*, 1997; Hajnoczky *et al.*, 1999).

The present results resuscitate an old question: do mitochondria contribute to the cytosolic Ca<sup>2+</sup> buffering in beating cardiac cells (Affolter *et al.*, 1976)? If this is the case, then mitochondrial Ca<sup>2+</sup> uptake and release might modify excitation-contraction coupling and, thus, the mechanical activity of cardiomyocytes. At present, the available data can not provide an answer to this question, because: (i) Ca<sup>2+</sup> probes measure the free Ca<sup>2+</sup> within the mitochondrial matrix, but the amount of calcium sequestered by mitochondria cannot be assessed in the absence of information concerning the ratio between the free and total forms of this cation within mitochondria; (ii) owing to impermeability of these cardiomyocytes to Ruthenium Red, the contribution of mitochondria can not be eliminated experimentally. This limitation is reinforced by the wide range of cellular effects, well beyond the inhibition of Ca<sup>2+</sup> transport, elicited by mitochondrial inhibitors or uncouplers.

Finally, the data presented here are also of relevance not only for cardiac physiology but, in general terms, for a better understanding of the role of mitochondrial Ca<sup>2+</sup> accumulation in cell physiology. Up until now, mitochondrial Ca<sup>2+</sup> handling has been shown to be involved in relatively slow processes such as stimulation of G protein-coupled receptors or prolonged opening of Ca<sup>2+</sup> channels (Rizzuto *et al.*, 1993; Montero *et al.*, 2000; Szalai *et al.*, 2000). The present findings directly demonstrate that brief, physiologically relevant stimuli, such as cardiac action potentials, result in a significant Ca<sup>2+</sup> accumulation by mitochondria. They also reveal that the frequency and amplitude of mitochondrial Ca<sup>2+</sup> changes can be modulated by physiological stimuli. In light of this, the kinetics of mitochondrial Ca<sup>2+</sup> handling appear perfectly suited to cope with the rapid [Ca<sup>2+</sup>]<sub>c</sub> changes (in the few millisecond range) that occur not only in cardiomyocytes, but also in many other excitable cells, primarily neurones.

## Materials and methods

### Cell culture

Cultures of cardiomyocytes were prepared from ventricles of Wistar rats (0–3 days after birth) as previously described (Dostal *et al.*, 1992). The heart was removed and rapidly placed in ice-cold HEPES-buffered saline containing 116 mM NaCl, 0.8 mM NaH<sub>2</sub>PO<sub>4</sub>, 5.3 mM KCl, 0.4 mM MgSO<sub>4</sub>, 5 mM glucose and 20 mM HEPES pH adjusted to 7.35. Atria were discarded and ventricles were submitted to four subsequent digestions with 0.45 mg/ml collagenase and 5% pancreatin. The first supernatant containing cell debris and blood cells was discarded. Cell suspensions from the three subsequent digestions (20 min each) were centrifuged and the pellet was resuspended in 2 ml of newborn calf serum. Cells were pooled and resuspended in a complete medium DMEM/Medium 199 (4:1) supplemented with 2 mM glutamine, penicillin (100 U/ml), streptomycin (100 mg/ml), 5% newborn calf serum and 10% horse serum. Cells were then incubated for 1 h at 37°C, allowing a selective attachment of non-myocytes. The unattached cells were plated at a density of 0.6 × 10<sup>6</sup> cell/ml onto 13 or 24 mm diameter coverslips coated with laminin (1 µg/cm<sup>2</sup>). The first day of culture, cells were washed and maintained in DMEM/Medium 199 (4:1) medium containing 5% horse serum and 0.5% newborn calf serum for 3 days. HeLa cells were grown in DMEM medium supplemented with 10% fetal calf serum in 75 cm<sup>2</sup> Falcon flasks. Two days before transfection, cells were seeded onto 13 mm diameter coverslips to be used for Ca<sup>2+</sup> measurement.

### cDNA constructs and transfection

The different constructs have been described in detail previously (Rizzuto *et al.*, 1993, 1996; Nagai *et al.*, 2001). Transfections were carried out on the first day of culture using the FuGENE 6 reagent (Roche). For a 13 mm diameter coverslip, 1.5 µg of DNA was mixed with 3 µl of Fugene reagent. All the volumes were doubled for transfection of cells plated onto 24 mm diameter coverslips. All the experiments were then performed at day 3 after transfection.

### Aequorin measurements

For the cells transfected with the cyt-Aeq and the mit-Aeq constructs, reconstitution of the functional aequorin was carried out in a modified Krebs-Ringer buffer (125 mM NaCl, 5 mM KCl, 1 mM Na<sub>2</sub>PO<sub>4</sub>, 1 mM MgSO<sub>4</sub>, 5.5 mM glucose and 20 mM HEPES pH 7.4) in the presence of either 100 µM EGTA or 1 mM CaCl<sub>2</sub> supplemented with 1% serum and 5 µM coelenterazine for 1 h at 37°C. After the reconstitution step, the cells were placed in a perfused, thermostated chamber in close proximity to a low noise photomultiplier, with a built in amplifier discriminator. The output of the discriminator was captured by a Thorn-EMI photon counting board and stored in an IBM compatible computer for further analysis. Aequorin photon emission was calibrated off line into [Ca<sup>2+</sup>] values using a computer algorithm based on the Ca<sup>2+</sup> response curve, as described previously (Brini *et al.*, 1995). While in previous experiments light output was integrated for 1 s, in all the present experiments (except those shown in Figure 1C and D) the maximum speed of data collection of the apparatus was employed, i.e. collection every 50 ms. The calibration was modified accordingly. When typical traces are presented they are



representative of experiments carried out on at least three different cell cultures. Possible interference of oxygen radicals on aequorin luminescence was verified by treating the cells with the cell permeant antioxidant Trolox (10  $\mu$ M) that has been proven previously to prevent oxygen radical effects on indo-1 fluorescence (Scheenen *et al.*, 1996). No effect of this compound on the kinetics of aequorin luminescence was observed.

#### **Determination of the subcellular distribution of recombinant aequorin**

After reconstitution with coelenterazine according to the standard protocol, the cells were treated for 5 min with 50  $\mu$ M digitonin in  $\text{Ca}^{2+}$ -free medium containing 1 mM EGTA and the first supernatant was collected. The same cells were further treated for 15 min with 0.1% Triton X-100 and the second supernatant was collected. The aequorin and the enzyme markers released in each supernatant were measured according to standard procedures. The activity of lactate dehydrogenase and of citrate synthase were followed spectrophotometrically (Martin and Denton, 1970). Light emission of aequorin was measured with a Labtech luminometer after the addition of 2 mM  $\text{CaCl}_2$ .

#### **Determination of $[\text{Ca}^{2+}]$ changes with the ratiometric-pericams**

Cells expressing the mitochondrial and nuclear ratiometric-pericams were analyzed by videoimaging using the Till Photonics GmbH system. Cells were illuminated alternatively for 40 and 20 ms at 415 and 480 nm and the emitted light was filtered at 510 nm. The ratio images were calculated off line (1 ratio image every 92 ms) in different regions of interest corresponding to groups of mitochondria and/or the nucleoplasm.

#### **Immunocytochemistry and GFP fluorescence**

Cells plated on gelatin-coated glass coverslips were fixed with 4% formaldehyde for 30 min. Fixed cells were permeabilized with Triton X-100 0.5% for 5 min and were subsequently labeled with the anti-HA1 mouse monoclonal antibody 12CA5 (Balco, Berkeley CA) and the rabbit anti-mouse FITC (Vector Laboratories, Burlingame CA). Immunostained cells or cells expressing GFP were observed with a Bio-Rad confocal microscope.

#### **Statistical analysis**

Data are reported as means  $\pm$  S.D. Statistical differences were evaluated by a one-tailed Student's *t*-test, with a *p* value <0.05 being considered statistically significant.

## **Acknowledgements**

We are grateful to M.Zaccolo and P.Magalhães for help with microscope analyses and helpful discussions. We thank M.Colella, R.Menabo, G.Ronconi and M.Santato for technical assistance, F.Marotte for advice on the preparation of neonatal cardiac cells, and J.Alvarez for providing the computer program used in the analysis of aequorin light emission. This work was supported by grants from Telethon numbers 845 and 1226, from the Italian Association for Cancer Research (AIRC) ACC990092 and 98130471, the UE (2000-934), the Italian Minister of University and Scientific Research (MURST), the Italian CNR 'Target Project Biotechnology', the Armenise Harvard Foundation, and the European Society of Aldosterone Council to Tullio Pozzan and a grant from Italian CNR 9902453.CT04 to F.d.L.

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Received April 17, 2001; revised July 9, 2001;  
accepted July 18, 2001