# Histamine induces oscillations of mitochondrial free $Ca^{2+}$ concentration in single cultured rat brain astrocytes

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1. The free $Ca^{2+}$ concentration of mitochondria ($[Ca^{2+}]_m$) in cultured rat brain astrocytes was measured with a fluorescent $Ca^{2+}$ indicator, rhod-2, and laser confocal microscopy.

2. Confocal images revealed a rhod-2 distribution that matched mitochondrial localization.

3. Using a $Ca^{2+}$ ionophore, ionomycin, to clamp the $[Ca^{2+}]_m$ from 0 to 100 $\mu$M in order to obtain the minimal and maximal fluorescence of rhod-2 *in situ*, a $3{\cdot}5 \pm 0{\cdot}4$-fold increase in fluorescence intensity was observed, suggesting that the fluorescence of intramitochondrial rhod-2 was responding in a $Ca^{2+}$-sensitive manner, thereby allowing measurements of $[Ca^{2+}]_m$ in single astrocytes.

4. Exposure of fura-2-loaded astrocytes to 100 $\mu$M histamine produced a rapid and transient increase in cytosolic $Ca^{2+}$ concentration ($[Ca^{2+}]_c$) that lasted for several tens of seconds. The spike in $[Ca^{2+}]_c$ was frequently followed by variable numbers of repetitive oscillations of $Ca^{2+}$, which appeared to dampen in amplitude with time.

5. This pattern of histamine-induced $[Ca^{2+}]_c$ oscillations was also observed in rhod-2-loaded cells suggesting that $[Ca^{2+}]_m$ fluctuated with a similar frequency.

6. The oscillations of $[Ca^{2+}]_m$, but not of $[Ca^{2+}]_c$, were abolished by a proton ionophore, carbonyl cyanide m-chlorophenyl-hydrazone (CCCP), and by Ruthenium Red, a mitochondrial $Ca^{2+}$-uniporter inhibitor.

7. These results suggest that the mitochondrial $Ca^{2+}$ transport systems in cultured rat brain astrocytes are able to relay receptor-mediated $[Ca^{2+}]_m$ oscillations into mitochondria.

The importance of mitochondria in contributing to the spatio-temporal aspects of intracellular $Ca^{2+}$ signalling under physiological conditions is still controversial (for a recent review see Gunter, Gunter, Sheu & Gavin, 1994). Several previous studies using electron-probe microanalysis (EPMA) have suggested that the mitochondrial $Ca^{2+}$ content is very low and does not respond to physiological fluctuations in cytosolic $Ca^{2+}$ concentration ($[Ca^{2+}]_c$; Somlyo, Bond & Somlyo, 1985; Moravec & Bond, 1992). In addition, studies in isolated mitochondria have shown that the $Ca^{2+}$ uniporter has an affinity for $Ca^{2+}$ in the range 10–100 $\mu$M, which is approximately two orders of magnitude higher than the range of $[Ca^{2+}]_c$ changes that occur under physiological conditions (for review see Carafoli, 1987). Based on such observations, it has been concluded that mitochondria play a minor role, or no role at all, in the control of $[Ca^{2+}]_c$. However, this conclusion has been challenged recently by a number of experimental observations. First, in isolated guinea-pig ventricular myocytes stimulated with paired voltage-clamp pulses, a substantial amount of $Ca^{2+}$ was taken up during early systole and released during late systole and diastole, as detected by EPMA (Wendt-Gallitelli & Isenberg, 1991; Isenberg, Han, Schiefer & Wendt-Gallitelli, 1993). Second, in recent studies using recombinant aequorin targeted to mitochondria in bovine endothelial cells, it was observed that activation of the $P_{2Y}$ purinoceptor by exogenous ATP induced a rapid and transient increase in mitochondrial free $Ca^{2+}$ concentration ($[Ca^{2+}]_m$; Rizzuto, Simpson, Brini & Pozzan, 1992). Additional studies indicated that this dynamic change in $[Ca^{2+}]_m$ also occurred in other cell types such as the HeLa cell line and a pancreatic $\beta$-cell line (Rutter, Theler, Murgia, Wollheim, Pozzan & Rizzuto, 1993; Rizzuto, Bastianutto, Brini, Murgia & Pozzan, 1994). This dynamic variation of $[Ca^{2+}]_m$ in response to the rapid $[Ca^{2+}]_c$ fluctuations has also been implicated as the cause of oscillations in the mitochondrial membrane potential (Loew, Carrington, Tuft & Fay, 1994). These studies have thus provided new evidence that contradicts the previous notion that $[Ca^{2+}]_m$ does not readily follow the changes in $[Ca^{2+}]_c$ under physiological conditions. However, not all recent studies have reached the same conclusion. For instance, using $Mn^{2+}$

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to quench the fluorescence of cytosolic indo-1, to permit measurements of $[Ca^{2+}]_m$ in rat ventricular myocytes, it was observed that $[Ca^{2+}]_m$ increased with pacing frequency but failed to vary on a beat-to-beat basis (Miyata, Silverman, Sollott, Lakatta, Stern & Hansford, 1991). This obervation that $[Ca^{2+}]_m$ is dependent on the average $[Ca^{2+}]_c$ and independent of the frequency of stimulation was also demonstrated in isolated mitochondria from rat heart (Leisey, Grotyohann, Scott & Scaduto, 1993). Therefore, the question as to whether mitochondria can sequester $Ca^{2+}$ during physiological $[Ca^{2+}]_c$ transients is not yet fully resolved.

In the present study, the relationship between $[Ca^{2+}]_m$ and $[Ca^{2+}]_c$ was investigated. Two experimental techniques were combined to monitor $[Ca^{2+}]_m$. First, a cationic fluorescent probe, rhod-2 (Minta, Kao & Tsien, 1989), composed of a voltage-sensitive rhodamine molecule (Johnson, Walsh & Chen, 1980) attached to the $Ca^{2+}$ chelator BAPTA, was used. This dye has been shown to localize preferentially inside the mitochondria due to their negative membrane potential, thereby allowing $[Ca^{2+}]_m$ to be measured (Jou, 1992; Jou & Sheu, 1994; Mix, Drummond, Tuft & Fay, 1994; Sheu & Jou, 1994; Tsien & Bacskai, 1995). Second, confocal microscopy was used, allowing the 'visualization' of $[Ca^{2+}]_m$ in single cells or in single mitochondria (Jou & Sheu, 1994; Tsien & Bacskai, 1995). To induce $[Ca^{2+}]_c$ oscillations, cultured astrocytes were exposed to 100 $\mu$M histamine (Inagaki, Fukui, Ito, Yamatodani & Wada, 1991). A preliminary report of this work has appeared in abstract form (Jou & Sheu, 1994).

## METHODS

### Cell preparation

Cells from a rat brain astrocytic cell line (RBA-1) were used in this study. This cell line originated from a primary astrocyte culture of neonatal rat cerebrum and was naturally developed through successive cell passages (Jou, Jou, Chen & Lee, 1985). Staining of RBA-1 with the astrocyte specific marker, glial fibrillary acid protein, showed over 90% positive staining. The cells were grown in medium consisting of Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum. All cells were plated onto 25 mm-diameter glass coverslips for experiments (Model #1, VWR Scientific, San Francisco, CA, USA).

### Fluorescence measurement

Cells were loaded with the ester form of rhod-2 (1 $\mu$M) for 5 min at 37 °C in DMEM. The cells were then washed with Hepes-buffered solution containing (mM): KCl, 5; NaCl, 140; $CaCl_2$, 2; $MgCl_2$, 1; glucose, 10; Hepes, 5 (at pH 7·4); and incubated for 1 h to allow conversion of the dye to its $Ca^{2+}$-sensitive, free-acid form. The coverslips with dye-loaded cells were mounted in a modified Sykes-Moore Tissue Chamber (Bellco, Vineland, NJ, USA) and secured on the stage of an ACAS 570 laser photometer (Meridian Instruments, Okemos, MI, USA). Rhod-2 was excited at 514 nm from an argon ion laser with a beam diameter of 1 $\mu$m. The emission light above 575 nm was detected with a photomultiplier tube and digitized. With a set of pinhole apertures in front of the photomultiplier, the contaminating light from out-of-focus layers was removed to allow specific recording of the fluorescence signal in the mitochondria. Laser light-induced photobleaching of the dye and damage to the cell was minimized by using a scanning laser power of $\leqslant 10\%$ and by setting neutral density filters to allow only 1–10% of the light to be transmitted. The signal-to-noise ratio was improved by selecting an appropriate sensitivity of the photomultiplier tube and by averaging 64–128 samples from the same pixel. Because the peak of the rhod-2 fluorescence spectra does not shift upon $Ca^{2+}$ binding, it was not possible to apply the ratiometric method for quantitative determinations of $[Ca^{2+}]_m$. Therefore, the information derived from the measurements of rhod-2 fluorescence relates to the relative degree of $[Ca^{2+}]_m$ change and not to the absolute magnitude of change. For non-confocal whole-cell fluorescence measurements, such as $[Ca^{2+}]_c$ measurements with fura-2, an inverted microscope (Nikon Diaphot) equipped for epifluorescence was used (Delta-Scan 1; Photon Technology International, Brunswick, NJ, USA). The system allowed excitation of the cell at ultraviolet wavelengths (for fura-2) or at visible-light wavelengths (for rhod-2) through monochromators. The emission light was focused onto the photocathode of a photomultiplier tube operating as a photon counting device (R928; Hamamatsu, Japan). All experiments were performed at room temperature (20–22 °C).

All chemicals were obtained from Sigma; rhod-2 was purchased from Molecular Probes.

## RESULTS

Figure 1*A* shows a pseudocolour fluorescence image of an RBA-1 cell loaded with rhod-2. The image shows that rhod-2 was localized in distinct spots with a globular or filamentous shape. No fluorescence signal was detected in the cytosol (C) or nucleus (N). These fluorescence spots have an average diameter of approximately 1 $\mu$m and occupy approximately 20–30% of cytosolic area, consistent with the fluorescence signals originating in mitochondria. The distribution of fluorescence intensity, indicated by the psuedocolour spectrum, appeared to be heterogeneous. Because of the non-ratiometric recording of rhod-2 and the limitation of the spatial resolution (0·25 $\mu$m resolution for $x$- and $y$-axis and 0·70 $\mu$m resolution for $z$-axis), this heterogeneity could be attributed to a variety of factors that affect the fluorescence intensity, which include differences in the concentration of rhod-2, the plane of focus, and the thickness of mitochondria.

For monitoring $[Ca^{2+}]_m$ from a single mitochondrion, individual mitochondria were first selected as shown in the square box of Fig. 1*A*. These three mitochondria are magnified and displayed in Fig. 1*B*. The fluorescence was then recorded through a line scan, as indicated by the horizontal white line, at millisecond intervals. The fluorescence intensity profile from the line scan was plotted (Fig. 1*C*), showing that the rhod-2 signal was detected only in the mitochondrion and not in the adjacent cytosol. The slight distortion of this intensity profile from a rectangular shape is probably due to several factors such as blurring and shading effects, and a thinner diameter at the edge of mitochondria. Additional experiments designed to show that these fluorescence spots originated from intracellular compartments were performed: (1) photobleaching the

fluorescence in mitochondria with stronger laser light was not followed by a recovery of fluorescence in these spots; (2) permeabilizing the plasma membrane with digitonin (10 $\mu$M for 5 min; Borzak, Kelly, Kramer, Matoba, Marsh & Reers, 1990), which selectively released dye from the cytosol, did not reduce the fluorescence signal in these spots; and (3) quenching cytosolic rhod-2 with $Mn^{2+}$ (Miyata *et al.* 1991) did not alter the fluorescence intensity in these spots.

It has been shown recently that the potential-dependent J-aggregate-forming lipophilic cation JC-1 is a fluorescent marker for mitochondria (Smiley *et al.* 1991). Figure 2*A* shows a transmitted-light image of a group of three cells in contact with each other that were loaded with JC-1. The circular organelle at the centre of each cell is the nucleus with a diameter of approximately 10 $\mu$m. The darker spots, spreading throughout the cytosol with a diameter of approximately 1 $\mu$m, could be identified visually as mitochondria. This was further confirmed by the fluorescence image in Fig. 2*B*, which shows that the location of these darker spots coincided with the location of JC-1. Similarly, in rhod-2-loaded cells, it was verified, through video-displayed microscopic images, that the positions of visually identified mitochondria coincided with the positions of rhod-2 fluorescence.

For accurate $[Ca^{2+}]_m$ measurements, rhod-2 must not only be trapped inside the mitochondria, it must also be in a $Ca^{2+}$-sensitive form. A $Ca^{2+}$ ionophore ionomycin (5 $\mu$M) was used to 'clamp' the $[Ca^{2+}]_m$ from 0 to 100 $\mu$M to obtain minimal and maximal fluorescence of rhod-2 *in situ*. A 3·5 ± 0·4-fold increase in fluorescence intensity was observed

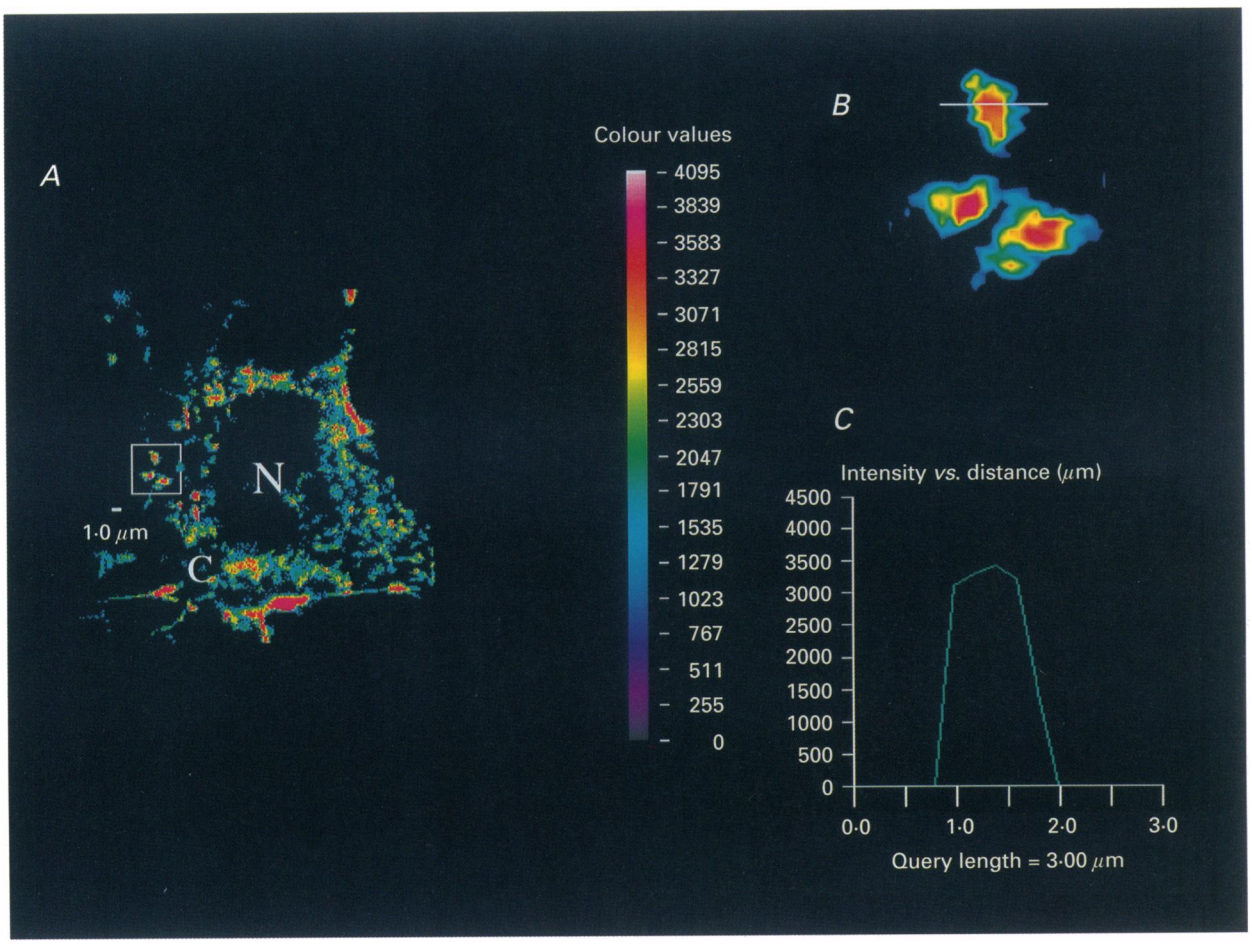


**Figure 1. Pseudocolour fluorescence image of a rhod-2-loaded RBA-1 cell**

*A*, rhod-2 was localized in punctate spots that coincided with the mitochondrial distribution. The approximate size of mitochondria is 1 $\mu$m, as indicated by the calibration bar. Note that the fluorescence signal was not detected in the cytosol (C) and nucleus (N). The rainbow spectrum indicates the fluorescence intensity (arbitrary units). *B*, illustration of line scan mode to measure fluorescence intensity from a single mitochondrion. Three mitochondria inside the white box in *A* were selected and magnified. The line scan mode was made by scanning laser light through the marked white line in this figure with a step size of 0·1 $\mu$m. *C*, fluorescence intensity profile from the line scan. The abscissa indicates the distance between the beginning and the end of the scanning line (query length) and the ordinate indicates the fluorescence intensity (arbitrary units). The intensity of fluorescence increased sharply upon scanning through the mitochondrion.

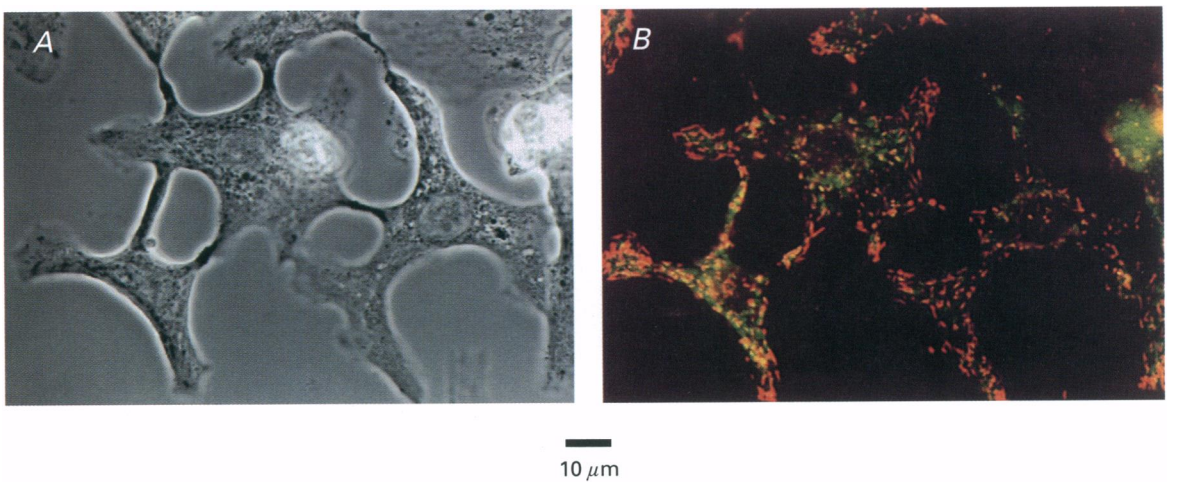


**Figure 2. Identification of mitochondrial locations with transmitted-light and fluorescence microscopy**

The darker spots in the transmitted-light image (*A*) could be visually identified as mitochondria. The location of these spots matches precisely with the location of JC-1, a voltage-sensitive fluorescent marker for mitochondria (*B*); note that there are two populations of mitochondrial membrane potential as indicated by green–yellow and orange–red fluorescence.

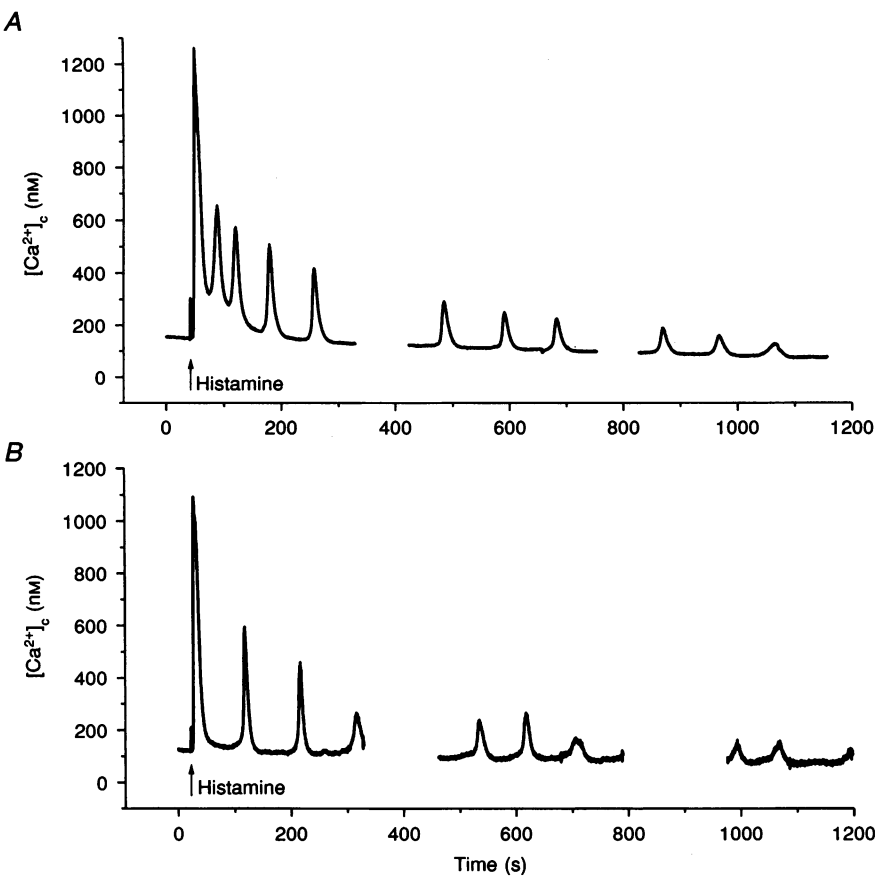


**Figure 3. Histamine-induced oscillations of $[Ca^{2+}]_c$ in RBA-1 cells**

*A*, addition of histamine (100 μM) to an RBA-1 cell induced a series of repetitive oscillations of $[Ca^{2+}]_c$, which appeared to dampen in amplitude with time. *B*, histamine-induced $[Ca^{2+}]_c$ oscillations in another cell with lesser $Ca^{2+}$ spikes.

(mean ± S.E.M., $n = 4$), suggesting that mitochondrial rhod-2 was indeed $Ca^{2+}$ sensitive. In the same range of $[Ca^{2+}]$ changes with rhod-2 free acid in solution, a 3·4-fold increase in fluorescence intensity was observed previously (Minta *et al.* 1989).

It has been shown that activation of histamine $H_1$ receptors in astrocytes causes an elevation of $[Ca^{2+}]_c$ via formation of inositol 1,4,5-trisphosphate (Inagaki *et al.* 1991). To confirm this observation in the present study, RBA-1 cells were loaded with fura-2 for $[Ca^{2+}]_c$ measurements. Figure 3*A* shows that addition of histamine (100 μM) to RBA-1 cells induced a rapid increase in $[Ca^{2+}]_c$ from a basal value of approximately 100 nM to a peak of 1 μM, which then gradually declined back to basal levels. The sharp spike in $[Ca^{2+}]_c$ was usually followed by a series of repetitive oscillations of $[Ca^{2+}]_c$ which dampened in amplitude with time. The frequency and amplitude of subsequent $Ca^{2+}$ spikes varied from cell to cell, as indicated in Fig. 3*B*. The duration of each $Ca^{2+}$ spike lasted a few tens of seconds. These histamine-induced $[Ca^{2+}]_c$ oscillations resulted mainly from the mobilization of $Ca^{2+}$ from intracellular stores as they persisted in a $Ca^{2+}$-free medium.

The next question examined was whether mitochondria could respond to histamine-induced changes in $[Ca^{2+}]_c$. Figure 4*A* shows a confocal image recorded from a portion of a rhod-2-loaded astrocyte in the control solution. To reduce cell injury resulting from phototoxicity, only ~20% of the entire cell (~10 μm × 10 μm) was imaged at a frequency of one recording per fifteen seconds or longer. This image was recorded through a ×100 water-immersion objective lens with a numerical aperture of 1·3 (compared with the ×40 water-immersion objective lens with a similar numerical aperture used in Fig. 1). At the scanning speed set in this experiment, it took 2 s to acquire an image. Four images, taken at time 0, 58, 116 and 174 s, were digitized and used to determine the baseline fluorescence in the control solution (Fig. 4*D*). Upon addition of histamine (100 μM) at time 176 s, $[Ca^{2+}]_m$ increased as shown in the images obtained at 214 s (Fig. 4*B*) and 229 s (Fig. 4*C*). At time 320 s, a smaller transient increase in $[Ca^{2+}]_m$ appeared (Fig. 4*D*). It should be noted that there is a small variability among the sequential images due to the movement of mitochondria, as reported previously (Johnson *et al.* 1980)

The above fluorescence images provided evidence for $[Ca^{2+}]_m$ changes upon histamine stimulation. However, prolonged recordings could not be obtained because of problems associated with photobleaching and phototoxicity, movement of mitochondria and variability in response from different

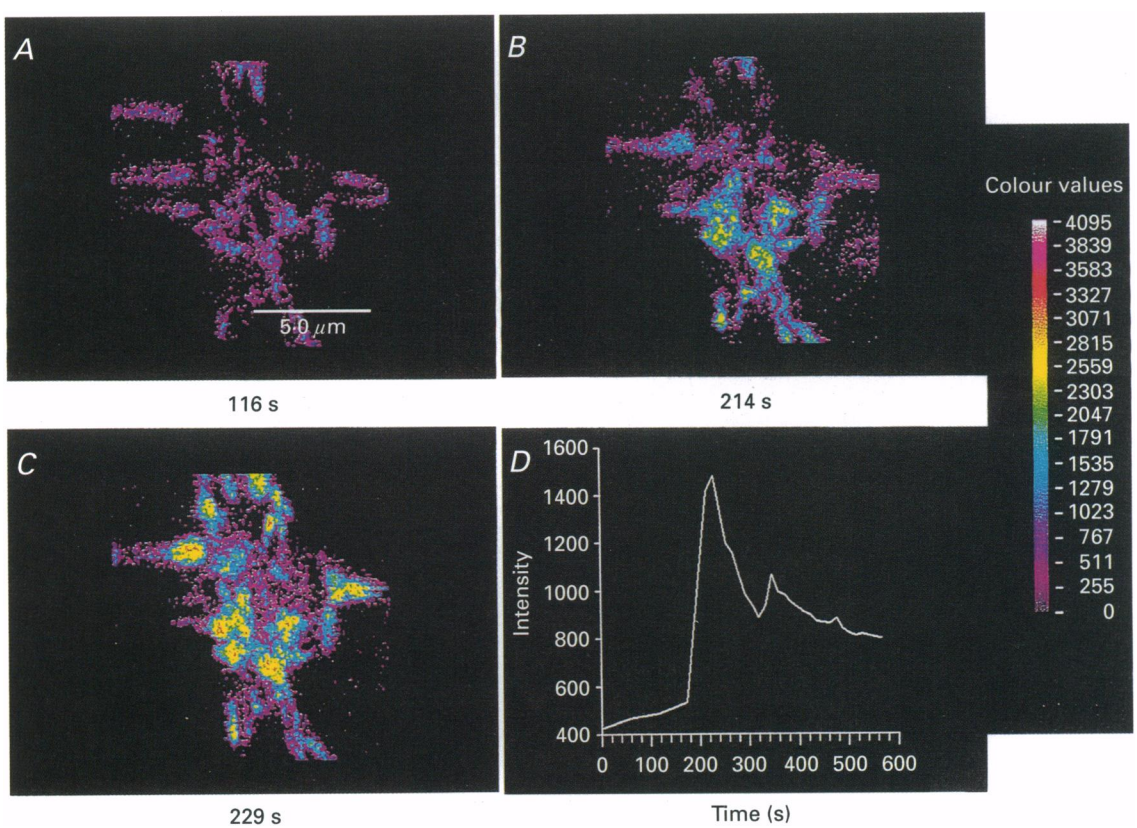


**Figure 4. Confocal images of $[Ca^{2+}]_m$ recorded from one-fifth of the area of an RBA-1 cell during histamine exposure**

$[Ca^{2+}]_m$ images in control solution (*A*) and histamine-containing solution (100 μM; *B* and *C*). The rainbow spectrum indicates the fluorescence intensity (arbitrary units). *D*, time course of $[Ca^{2+}]_m$ change. The ordinate indicates the fluorescence intensity (arbitrary units).

mitochondria. To compensate, we measured long-term fluorescence from entire areas of single cells loaded with rhod-2 using a non-confocal fluorescence microscope. Figure 5*A* shows a representative experiment from such a whole-cell fluorescence recording. The overall pattern of $Ca^{2+}$ oscillation was similar to $[Ca^{2+}]_c$. However, the time to peak of the first $Ca^{2+}$ spike was 2-fold longer for $[Ca^{2+}]_m$ than for $[Ca^{2+}]_c$ (3·4 ± 0·7 and 6·8 ± 1·0 s for $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$, respectively; means ± S.E.M., $n = 7$). In addition, the time of recovery to the basal level was significantly longer for $[Ca^{2+}]_m$ than for $[Ca^{2+}]_c$ (Fig. 5*B*). The peak intensity of the first spike was 1·5–2·0-fold higher than the control value.

The ability of mitochondria to sequester $Ca^{2+}$ depends on two major interrelated factors: (1) the negative mitochondrial membrane potential, serving as the driving force for $Ca^{2+}$ influx, and (2) a uniporter which transports $Ca^{2+}$ more selectively than other divalent cations (Gunter *et al.* 1994). Therefore, one can inhibit mitochondrial $Ca^{2+}$ uptake by either collapsing the membrane potential with protonophores such as carbonyl cyanide m-chlorophenyl-hydrazone (CCCP) or inhibiting the $Ca^{2+}$ uniporter with blockers such as Ruthenium Red. Figure 6*A* shows the effect of CCCP on $[Ca^{2+}]_c$. The addition of CCCP (2 μM) caused a small increase in $[Ca^{2+}]_c$. However, histamine-induced $[Ca^{2+}]_c$ oscillations persisted. In contrast, the addition of CCCP to rhod-2-loaded cells abolished the oscillations of $[Ca^{2+}]_m$ evoked by the addition of histamine (Fig. 6*B*). Similarly, inhibition of the $Ca^{2+}$ uniporter with Ruthenium Red (40 μM) did not block histamine-induced $[Ca^{2+}]_c$ oscillations but completely abolished $[Ca^{2+}]_m$ oscillations (Fig. 6*C* and *D*). These results suggest that the rhod-2 fluorescence originates from a CCCP- and Ruthenium Red-sensitive $Ca^{2+}$ pool.

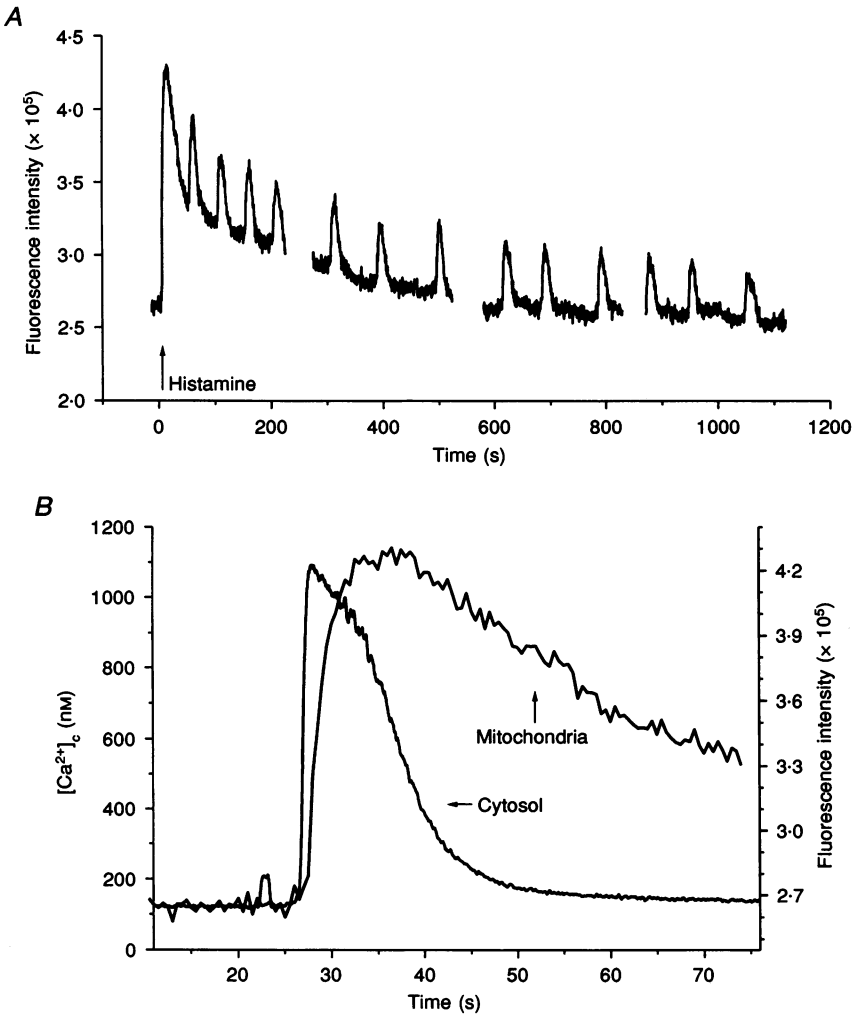


**Figure 5. Histamine-induced oscillations of $[Ca^{2+}]_m$ in an RBA-1 cell**

*A*, addition of histamine (100 μM) to an RBA-1 cell induced a series of repetitive oscillations of $[Ca^{2+}]_m$. *B*, superimposed $Ca^{2+}$ spikes from a fura-2- and a rhod-2-loaded cell. The fluorescence intensity is given in arbitrary units.

## DISCUSSION

The present study demonstrates that: (1) $[Ca^{2+}]_m$ *in situ* can be selectively monitored either in single cells or in single mitochondria using rhod-2 and confocal microscopy; and (2) mitochondria in cultured RBA-1 cells sequester cytosolic $Ca^{2+}$ during histamine-induced $Ca^{2+}$ oscillations.

### Measurement of $[Ca^{2+}]_m$ *in situ*

Techniques for measuring $[Ca^{2+}]_m$ in living cells have recently been described (Williford, Sharma, Korth & Sheu, 1990; Miyata *et al.* 1991; Jou, 1992; Rizzuto *et al.* 1992, 1994; Sheu & Jou, 1994). These developments have made it possible to investigate the regulation of mitochondrial $Ca^{2+}$ under physiological conditions. Nevertheless, these studies have several limitations. The use of conventional fluorescence digital-imaging microscopy with ratiometric indicators such as fura-2 (Williford *et al.* 1990) detects signals from out-of-focus areas so that fluorescence from mitochondria would be contaminated by that from the cytosol. Additionally, it has been shown that fura-2 binds to cytosolic proteins and that this changes the kinetic and spectral properties of the indicator causing an artifactual heterogeneity in $[Ca^{2+}]_c$ distribution in skeletal muscle (Baylor & Hollingworth, 1988). The use of $Mn^{2+}$ to quench the cytosolic fluorescence provided a reasonable alternative method for measuring $[Ca^{2+}]_m$ without $[Ca^{2+}]_c$ contamination (Miyata *et al.* 1991). However, $Mn^{2+}$ severely disturbs mitochondrial $Ca^{2+}$ transport mechanisms (Gunter *et al.* 1994) and would therefore be expected to alter $[Ca^{2+}]_m$. Recent developments of fusing the complementary DNA for the $Ca^{2+}$-sensitive photoprotein aequorin in frame with DNA encoding a mitochondrial targeting sequence have provided a new opportunity for studying mitochondrial $Ca^{2+}$ homeostasis in intact cells (Rizzuto *et al.* 1992, 1994). Unfortunately, this method can only be applied in cultured cells due to the requirements of gene transfection. Moreover, because of limited efficiency of transfection, a group of 1000–10 000 cells are required for performing experiments (Rutter *et al.* 1993). This makes the study of $Ca^{2+}$ oscillations in single cells impossible because each cell does not oscillate $Ca^{2+}$

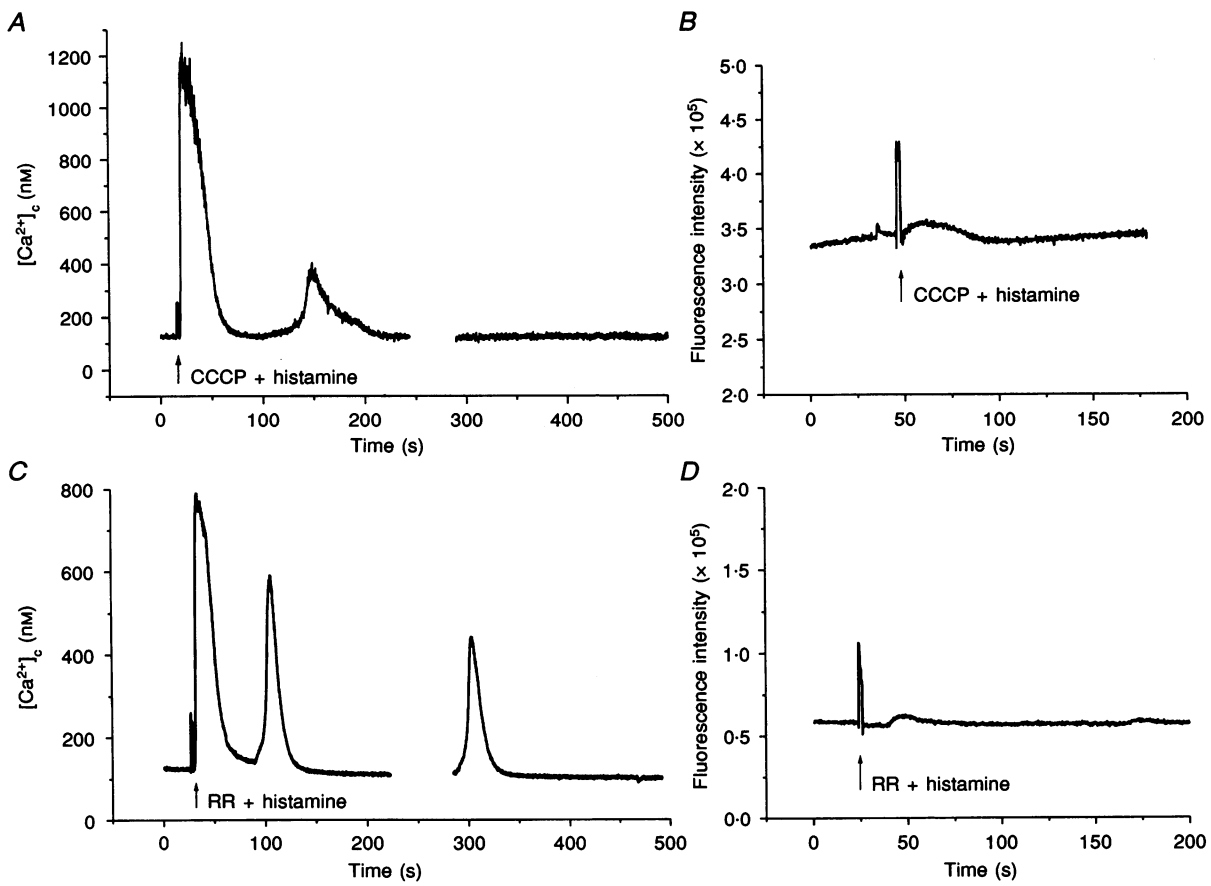


**Figure 6. Effects of CCCP and Ruthenium Red (RR) on the histamine-induced $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ oscillations**

*A*, histamine-induced $[Ca^{2+}]_c$ oscillations in the presence of CCCP (2 $\mu$M). *B*, CCCP abolished histamine-induced $[Ca^{2+}]_m$ oscillations. *C*, histamine-induced $[Ca^{2+}]_c$ oscillations in the presence of RR (40 $\mu$M). *D*, RR abolished histamine-induced $[Ca^{2+}]_m$ oscillations. In *B* and *D*, the fluorescence intensity is given in arbitrary units.

synchronously. Finally, aequorin has multiple $Ca^{2+}$-binding sites and once the protein has luminesced it can not do so again. Thus, quantitative determinations of the magnitude and kinetics of $Ca^{2+}$ transients with this technique are difficult.

The principle of using rhod-2 is similar to other commonly used $Ca^{2+}$ indicators such as fura-2 and indo-1. Therefore, the method described here should be easily applicable to studying mitochondrial $Ca^{2+}$ regulation under physiological and pathological conditions in all cell types (Tsien & Bacskai, 1995; Hajnoczky, Robb-Gaspers, Seitz & Thomas, 1995). The stronger fluorescence intensity of rhod-2 allows us to image $[Ca^{2+}]_m$ from single mitochondria in single cells, and thus provide the capacity for studying $Ca^{2+}$ regulation in individual mitochondria. In cardiac myocytes, two populations of mitochondria, subsarcolemmal and interfibrillar mitochondria, have been identified (Palmer, Tandler & Hoppel, 1977). These different populations of mitochondria have also been shown to possess different $Ca^{2+}$ regulatory properties (McMillian-Wood, Wolkowicz, Chu, Tate, Goldstein & Entman, 1980). The JC-1 image in Fig. 2 indicates that there are two populations of membrane potential across mitochondria in astrocytes: a less negative membrane potential (green–yellow fluorescence) and a more negative membrane potential (orange–red fluorescence). Since the uptake of $Ca^{2+}$ by mitochondria is voltage dependent, this heterogeneous distribution of mitochondrial membrane potential would have differential effects on mitochondrial $Ca^{2+}$ transport. This question is currently under investigation.

However, there are two major limitations in the present technique: first, the characteristics of the rhod-2 fluorescence spectra preclude the quantitative measurements of $[Ca^{2+}]_m$ by a ratiometric method; and second, the small size of mitochondria (1–2 $\mu$m) makes it difficult to obtain a distinct fluorescence image of a single mitochondrion with high spatial resolution. To overcome the limitation of indicators that do not allow for ratiometric determinations, a modified method has been described recently involving the simultaneous loading of fluo-3 and fura-red into cells (Lipp & Niggli, 1993). When excited at 488 nm, fluo-3 and fura-red have emission spectra that peak at 525 and 660 nm, respectively. These distinct wavelengths for peak fluorescence permit quantitative measurements of $[Ca^{2+}]_c$ by calculating the ratio of fluorescence of fluo-3 at 525 nm over fluorescence of fura-red at 660 nm. This method should also be applicable for future studies of $[Ca^{2+}]_m$ determination by loading rhod-2 and fura-red simultaneously into mitochondria. As for the limitation on the spatial resolution of $[Ca^{2+}]_m$ images, with a scanning step size of 0·1 $\mu$m, 12-bit digital conversion, and ×100 objective, the resolution of the $x$- and $y$-axis is 0·25 $\mu$m and the $z$-axis resolution is 0·70 $\mu$m for our confocal microscope. Therefore, for mitochondria with a thickness of 1 $\mu$m or less, the spatial resolution is particularly poor in the $z$-axis. This is likely to be the principal reason for the apparent heterogeneity of $[Ca^{2+}]_m$ distribution seen in Fig. 1.

### Mitochondrial $Ca^{2+}$ oscillations

Strong evidence has accumulated in the past decade suggesting that $Ca^{2+}$ acts as a second messenger in the mitochondria to regulate the rate of oxidative phosphorylation (for review, see Denton & McCormack, 1990; Hansford, 1994; Gunter *et al.* 1994). Paradoxically, it is also widely claimed that mitochondria contribute minimally, if at all, to the spatial and temporal dynamics of cytosolic $Ca^{2+}$ under physiological conditions (for review, see Carafoli, 1987). However, recent studies have shown that not only can mitochondria sequester $Ca^{2+}$ over the physiological range of $Ca^{2+}$ increases, but they can do so fast enough to take up $Ca^{2+}$ during a single $Ca^{2+}$ pulse (Thayer & Miller, 1990; Wendt-Gallitelli & Isenberg, 1991; Isenberg *et al.* 1993; Hajnoczky *et al.* 1995; LeMasters *et al.* 1995; Herrington, Park, Babcock & Hille, 1996; Budd & Nicholls, 1996). Consistent with these studies, the present results show that $[Ca^{2+}]_m$ oscillates, with a few seconds lag, concomitantly with $[Ca^{2+}]_c$ fluctuations in glial cells under histamine exposure. The observed lag could be due either to the fact that the mitochondrial uniporter has a lower $Ca^{2+}$ affinity, such that $[Ca^{2+}]_c$ has to rise above certain levels for its activation, or that it takes time for $Ca^{2+}$ to diffuse from the endoplasmic reticulum to mitochondria. The present observation, that the decline in mitochondrial $Ca^{2+}$ is slower than the cytosolic signal, differs from results obtained with aequorin targeted to mitochondria (Rizzuto *et al.* 1992). This discrepancy cannot be readily explained, although it could be the result of using different cells, different methodologies, or different temperatures. Consistent with our observation is a recent report showing that the rise in $[Ca^{2+}]_m$ is slower, smaller, but more prolonged than the transient rise in $[Ca^{2+}]_c$ (Babcock, Herrington & Hille, 1996).

The observation that CCCP and Ruthenium Red abolish oscillations associated with the rhod-2 but not fura-2 recorded fluorescence is consistent with the notion that the rhod-2 fluorescence originates from mitochondria. However, the frequency and persistence of $[Ca^{2+}]_c$ oscillations were also partially reduced under these conditions. This is probably due to the additional effects of CCCP and Ruthenium Red on other $Ca^{2+}$ transport mechanisms. For example, addition of CCCP could deplete cellular ATP and eventually affect general cellular $Ca^{2+}$ metabolism (Fulceri, Bellomo, Mirabelli, Gamberucci & Benedetti, 1991), and the extracellular application of Ruthenium Red blocks voltage-gated $Ca^{2+}$ currents (Duchen, 1992).

### Physiological implications of $[Ca^{2+}]_m$ oscillations

Several lines of evidence suggest that the mitochondrial $Ca^{2+}$ uptake systems serve to: (1) determine the kinetics of $[Ca^{2+}]_c$ transients in neurones and cardiomyocytes (Thayer

& Miller, 1990; Duchen, Valdeolmillos, O'Neill & Eisner, 1990; Isenberg *et al.* 1993; Friel & Tsien, 1994); (2) cycle $Ca^{2+}$ between sarcoplasmic reticulum and mitochondria in heart (Bassani, Bassani & Bers, 1993); and (3) regulate bioenergetic metabolism (Denton & McCormack, 1990; Hansford, 1994). The feasibility of monitoring $[Ca^{2+}]_m$ in living cells, as demonstrated in the present study, and potential improvements in quantitative approaches will help to explore further the physiological roles of mitochondrial $Ca^{2+}$ transport.


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