Mitochondrial Ca^{2+} homeostasis during Ca^{2+} influx and Ca^{2+} release in gastric myocytes from *Bufo marinus*

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1. The Ca^{2+}-sensitive fluorescent indicator rhod-2 was used to monitor mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_m$) in gastric smooth muscle cells from *Bufo marinus*. In some studies, fura-2 was used in combination with rhod-2, allowing simultaneous measurement of cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) and $[Ca^{2+}]_m$, respectively.

2. During a short train of depolarizations, which causes Ca^{2+} influx from the extracellular medium, there was an increase in both $[Ca^{2+}]_i$ and $[Ca^{2+}]_m$. The half-time ($t_{½}$) to peak for the increase in $[Ca^{2+}]_m$ was considerably longer than the $t_{½}$ to peak for the increase in $[Ca^{2+}]_i$. $[Ca^{2+}]_m$ remained elevated for tens of seconds after $[Ca^{2+}]_i$ had returned to its resting value.

3. Stimulation with caffeine, which causes release of Ca^{2+} from the sarcoplasmic reticulum (SR), also produced increases in both $[Ca^{2+}]_i$ and $[Ca^{2+}]_m$. The values of $t_{½}$ to peak for the increase in $[Ca^{2+}]$ in both cytoplasm and mitochondria were similar; however, $[Ca^{2+}]_i$ returned to baseline values much faster than $[Ca^{2+}]_m$.

4. Using a wide-field digital imaging microscope, changes in $[Ca^{2+}]_m$ were monitored within individual mitochondria *in situ*, during stimulation of Ca^{2+} influx or Ca^{2+} release from the SR.

5. Mitochondrial Ca^{2+} uptake during depolarizing stimulation caused depolarization of the mitochondrial membrane potential. The mitochondrial membrane potential recovered considerably faster than the recovery of $[Ca^{2+}]_m$.

6. This study shows that Ca^{2+} influx from the extracellular medium and Ca^{2+} release from the SR are capable of increasing $[Ca^{2+}]_m$ in smooth muscle cells. The efflux of Ca^{2+} from the mitochondria is a slow process and appears to be dependent upon the amount of Ca^{2+} in the SR.

Application of a contractile stimulus to smooth muscle produces an elevation in the free cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$), which subsequently leads to activation of the contractile machinery (Kamm & Stull, 1985). Depending upon the nature of the contractile stimulus, the increase in $[Ca^{2+}]_i$ may reflect influx of Ca^{2+} from the extracellular medium, and/or release of Ca^{2+} from the sarcoplasmic reticulum (SR). Influx of Ca^{2+} from the extracellular medium can occur through voltage-operated or receptor-operated Ca^{2+} channels in the plasma membrane (Benham & Tsien, 1987; Hess, 1990). Ca^{2+} may be released from the SR through the inositol 1,4,5-trisphosphate ($InsP_3$) receptor (Somlyo *et al.* 1985; Berridge, 1993) and/or the ryanodine receptor (Fabiato, 1989). While much attention has been given to the processes controlling Ca^{2+} entry into the cytoplasm, less is known about the processes which are responsible for restoring $[Ca^{2+}]_i$ to its resting level, thereby bringing about relaxation. As is the case for most other cells, several processes are thought to be involved in the removal of Ca^{2+} from the cytoplasm of smooth muscle cells. These are: the ATP-dependent Ca^{2+} pumps in the SR and plasma membrane (Missiaen *et al.* 1991); the plasma membrane Na^+–Ca^{2+} exchanger (McCarron *et al.* 1994); and the mitochondria (Drummond & Fay, 1996; McGeown *et al.* 1996).

The role of mitochondria in regulating $[Ca^{2+}]_i$ in smooth muscle cells remains an unsettled issue. While there are several studies indicating mitochondrial Ca^{2+} uptake during stimulation with high K^+ in smooth muscle (Karaki & Weiss 1981), there are others reporting no change in the level of mitochondrial Ca^{2+} during depolarization (Somlyo *et al.* 1979). We have previously shown that under conditions where mitochondrial Ca^{2+} uptake is impaired, by carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP) or cyanide, the rates of Ca^{2+} removal from the cytoplasm following depolarizing voltage steps were significantly reduced during tight-seal, whole-cell recording (Drummond & Fay, 1996). This effect did not appear to be due to ATP

insufficiency, since ATP was included in the patch pipette, and similar reduction of cytoplasmic Ca^{2+} removal was observed when Ruthenium Red was used to block the mitochondrial Ca^{2+} uniporter. Further indirect evidence that mitochondria are involved in Ca^{2+} homeostasis in smooth muscle cells has been provided by studies where the decay time constant of a Ca^{2+}-dependent Cl^- current was found to be prolonged under conditions where mitochondrial Ca^{2+} uptake was inhibited (Greenwood *et al.* 1997). More recently, direct demonstration of mitochondrial Ca^{2+} uptake in smooth muscle was provided by two studies, where mitochondrial $[Ca^{2+}]$ ($[Ca^{2+}]_m$) was monitored with the Ca^{2+} sensitive fluorescent indicator rhod-2 (Drummond & Tuft, 1999; McCarron & Muir, 1999). These studies revealed that release of Ca^{2+} from the SR (Drummond & Tuft, 1999), or influx from the extracellular medium through voltage-sensitive Ca^{2+} channels (McCarron & Muir, 1999), resulted in an increase in $[Ca^{2+}]_m$. Increases in $[Ca^{2+}]_m$ were also detected using rhod-2 during agonist stimulation of cultured smooth muscle cells (Monteith & Blaustein, 1999).

The objective of the present study was to employ recently developed methodology for monitoring $[Ca^{2+}]_m$, to substantiate and advance our previous pharmacological studies on the involvement of mitochondrial Ca^{2+} uptake during depolarizing stimulation of smooth muscle cells (Drummond & Fay, 1996; McGeown *et al.* 1996). Specifically, by using rhod-2 to monitor $[Ca^{2+}]_m$ directly, we set out to establish whether $[Ca^{2+}]_m$ increased following depolarization-induced stimulation of Ca^{2+} influx from the extracellular medium or caffeine-induced release of Ca^{2+} from the SR. Two approaches were employed. First, $[Ca^{2+}]_m$ was monitored using a high speed multi-wavelength micro-fluorimeter which allowed simultaneous monitoring of both $[Ca^{2+}]_m$ and $[Ca^{2+}]_i$ with high temporal resolution (Drummond & Tuft, 1999). This approach provided detailed kinetic information about the time course of the Ca^{2+} changes in each of these cellular compartments. Second, the use of a wide-field, high-speed, digital imaging microscope, allowed us to monitor changes in $[Ca^{2+}]_m$ at the level of individual mitochondria *in situ*. It was found that stimulation of either Ca^{2+} influx, or Ca^{2+} release from the SR were capable of producing an increase in $[Ca^{2+}]_m$. While $[Ca^{2+}]_i$ normally returned to baseline values within 20 s following either form of stimulation, $[Ca^{2+}]_m$ was restored to baseline values at a considerably slower rate which appeared to vary depending on the level of Ca^{2+} in the SR.

METHODS

Cell isolation

The methods used have been described in detail elsewhere (Fay *et al.* 1982; McGeown *et al.* 1996) and will only be described briefly herein. Adult toads (*Bufo marinus*) were killed by decapitation using a small animal guillotine, as approved by the University of Massachusetts Medical Center Animal Care Committee, following guidelines of the US Department of Agriculture, and Health and Human Services. Single smooth muscle cells were freshly isolated from the stomach by enzymatic dissociation as previously described (Fay *et al.* 1982; McGeown *et al.* 1996). Experiments were carried out using the following solutions. Extracellular (mM): NaCl, 94; KCl, 3; $MgCl_2$, 1; tetraethylammonium (TEA), 10; Hepes, 5; $CaCl_2$, 20; pH adjusted to 7·4 with NaOH. Pipette (mM): CsCl, 140; $MgCl_2$, 1; Na_2ATP, 3·6; Hepes, 20; pH adjusted to 7·15–7·2 with CsOH. All experiments were carried out at room temperature.

Simultaneous measurement of mitochondrial and cytoplasmic $[Ca^{2+}]$

To monitor $[Ca^{2+}]$ in the cytoplasm and mitochondria simultaneously, fluorescence was measured using a custom built multi-wavelength microfluorimeter (Drummond & Tuft, 1999). Briefly, the system consisted of a Zeiss IM-35 inverted microscope (Nikon ×40, 1·3NA) with a specially designed excitation path and photomultiplier tube (Thorn EMI type 9954A, Thorn EMI, Rockaway, NJ, USA). The excitation path comprised a series of dichroic mirrors and a 'chopper' wheel, which permitted excitation of up to four wavelengths every 20 ms. In the present study, the excitation wavelengths for fura-2 were 340 nm and 380 nm (~10 nm bandwidth), and for rhod-2 the excitation wavelength was 500 nm (~10 nm bandwidth). A 560 nm (~80 nm bandwidth) emission filter was used for both fura-2 and rhod-2 emission. An image mask was used to exclude the field beyond the cell, thereby reducing the background contribution to the fluorescence signal.

In order to monitor $[Ca^{2+}]_m$, isolated smooth muscle cells were incubated with the Ca^{2+} sensitive fluorescent indicator rhod-2 AM (1–1·5 μM), for at least 1 h at room temperature. Rhod-2 has been used by several different laboratories to monitor changes in $[Ca^{2+}]$ within the mitochondrial matrix, in many different cell types (Mix *et al.* 1994; Sheu & Jou, 1994; Rutter *et al.* 1996; Babcock *et al.* 1997; Trollinger *et al.* 1997; Drummond & Tuft, 1999; McCarron & Muir, 1999). Binding of Ca^{2+} to rhod-2 increases its fluorescence; however, since there is no significant shift in excitation or emission wavelengths upon Ca^{2+} binding, it cannot be used for ratio imaging. Thus, to avoid potential artifacts resulting from cell movement, contraction was blocked with wortmannin (100 μM in the extracellular solution) (Nakanishi *et al.* 1992) in all studies involving rhod-2. Comparison with previous studies in our laboratory indicated that wortmannin did not have any effect on the cytoplasmic Ca^{2+} signal in these cells. Due to uncertainties concerning the K_d of the indicator within the mitochondrial matrix, and the accuracy of establishing fluorescence in the absence of Ca^{2+} (F_{min}) and that in the presence of saturating Ca^{2+} (F_{max}), mitochondrial rhod-2 fluorescence was not converted to $[Ca^{2+}]$. Rather, rhod-2 fluorescence is reported as $100\Delta F/F_o$, where F is the measured fluorescence, and F_o is the fluorescence before application of either the depolarizing stimulus or caffeine. In discussing results, we refer to an increase or decrease in rhod-2 fluorescence as an increase or decrease in $[Ca^{2+}]_m$. $[Ca^{2+}]_i$ was monitored with fura-2 (50 μM), which was included as the free acid in the patch pipette solution. Fura-2 ratios were converted to $[Ca^{2+}]_i$ using the method described by Grynkiewicz *et al.* (1985) and an assumed Ca^{2+}–fura-2 K_d of 200 nM. The fluroescence ratio in saturating Ca^{2+} (R_{max}) and that in the absence of Ca^{2+} (R_{min}), and the fluorescence ratio (Ca^{2+} free/saturating Ca^{2+}) for dye excited at 380 nm (β) were determined as previously described (Drummond & Fay, 1996).

Imaging changes in $[Ca^{2+}]$ within individual mitochondria

Mitochondria labelled with rhod-2 (as described above) were imaged using an high-speed, wide-field digital imaging microscope (see

ZhuGe *et al.* 1999 for a complete description of this system). Briefly, the system is based on a custom built inverted microscope. An objective (Nikon × 60, 1·4 NA), which can be rapidly focussed by the 20 μm total travel piezoelectric translator, forms a real image on a 128 pixel × 128 pixel frame transfer charge-coupled device (CCD). The 128 × 128 image on the CCD is then transferred in 50 μs to the 128 × 128 storage frame, leaving the image frame cleared for the next image. The storage frame is then read out to computer memory in 1·8 ms, allowing a maximum image rate of 543 s^{-1}. The pixel size used in the present study was 333 nm × 333 nm, giving an area of coverage 42·6 μm × 42·6 μm. The CCD camera was developed in conjunction with Lincoln Laboratory at Massachusetts Institute of Technology (Lexington, MA, USA). The 514 nm line of a multi-line argon–krypton laser was expanded to provide wide-field illumination for rhod-2 excitation. A laser shutter controlled the exposure duration and a 550 nm long pass emission filter was used for rhod-2 emission. A rapid nine image through-focus series was taken at $Z = -2{\cdot}0, -1{\cdot}5, -1, -0{\cdot}5, 0, 0{\cdot}5, 1, 1{\cdot}5, 2{\cdot}0$ μm. Using 5–7 ms exposures, with 10 ms between exposures to allow for the piezoelectric translator to move the objective, meant that a nine image through-focus series could be acquired in 125–143 ms. A through-focus image set was acquired before stimulation; ten through-focus sets were then acquired at 1 s intervals commencing 0·5 s after initiating the depolarizing or caffeine stimulus. An additional nine through-focus sets were then acquired at 15 s intervals.

Image processing and data analysis

Although there should be no rhod-2 outside the cell boundary, and very little in the cytoplasm, fluorescence is seen in these regions because of blurred fluorescence from mitochondria beyond the plane of focus. Thus, image restoration is essential to enable accurate data analysis. To reverse the distortion, the data were processed using a constrained deconvolution algorithm based on regularization theory as described by Carrington *et al.* (1990). This requires that a quantitative calibration of the optical characteristics of the microscope be carried out. This is achieved by acquiring a 3-D image of a sub-resolution fluorescence bead (Molecular Probes, OR, USA). Prior to image restoration, the data are digitally corrected for background intensity and small heterogeneities in the CCD sensitivity (Carrington *et al.* 1990).

Image processing and data analysis were performed using custom designed software, running on a SiliconGraphics work station. The fluorescence of an individual mitochondrion is represented by the pixel of maximum intensity. The brightest pixel presumably is the most in-focus portion of the mitochondrion. Interactive analysis and visualization software enabled determination of the brightest pixel for each mitochondrion. Twenty to thirty mitochondria were randomly chosen for each cell and followed through time. Values for each mitochondrion were normalized to the pre-stimulus fluorescence value, and in turn averaged to obtain a measure of the fluorescence of mitochondria in response to depolarizing or caffeine stimulation.

Measurement of mitochondrial membrane potential

In order to monitor any change in mitochondrial membrane potential which occurs following influx of Ca^{2+} into the matrix, tetramethylrhodamine ethyl ester (TMRE) was used (Ehrenberg *et al.* 1988; Loew *et al.* 1994; Duchen *et al.* 1998). Smooth muscle cells were exposed to 100 nM TMRE for at least 10 min. At this concentration, the potential-dependent accumulation of TMRE within mitochondria produces a quench of fluorescence, which is relieved by redistribution of the dye when the mitochondrial membrane potential is depolarized: thus, an increase in TMRE fluorescence is indicative of mitochondrial depolarization. TMRE fluorescence was monitored using the multi-wavelength microfluorimeter described above, with the same excitation and emission filters as used for rhod-2. This approach allowed for simultaneous measurement of the mitochondrial membrane potential and $[Ca^{2+}]_i$.

Electrophysiology

Whole-cell membrane currents were recorded during all experimental protocols using thin walled borosilicate patch electrodes (3–5 MΩ) (World Precision Instruments, FL, USA) with an Axopatch-1D patch-clamp amplifier (Axon Instruments, Foster City, CA, USA). Following rupture of the membrane patch, 20–30 min were allowed for dialysis of the cytoplasm before commencing any experimental protocols. This extended period of dialysis facilitated removal of any residual rhod-2 from the cytoplasm. Data were digitally stored on an IBM compatible PC, at a frequency of 1 kHz after being filtered with a low-pass filter (200 Hz cut-off), for subsequent off-line analysis.

Reagents and data analysis

Rhod-2 AM, fura-2, and TMRE were obtained from Molecular Probes (Eugene, OR, USA). Rhod-2 AM and TMRE were prepared as 1 mM stock solutions in DMSO. Fura-2 was prepared as a 10 mM stock solution in ultra pure water. Wortmannin, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP), caffeine and all other reagents were from Sigma (St Louis, MO, USA). Wortmannin and FCCP were prepared as 100 mM and 1 mM stock solutions in DMSO, respectively. Caffeine was applied from a pressure ejection pipette, positioned approximately 100 μm from the cell, using a picospritzer II (General Valve, Fairfield, NJ, USA).

Data are shown as means ± standard error of the mean (S.E.M.), and n refers to the number of cells. Statistical tests of difference were made using Student's t tests for paired and unpaired observations. The Kolmogorov–Smirnov test was used to test for differences in the frequency histograms for depolarizing and caffeine stimulation. $P < 0{\cdot}05$ was considered to be statistically significant.

RESULTS

Mitochondrial labelling with rhod-2

Figure 1 shows a gastric smooth muscle cell from *Bufo marinus*, which has been incubated with 1 μM of the Ca^{2+} sensitive fluorescent indicator rhod-2 AM for 1 h at room temperature. Note the accumulation of rhod-2 within the mitochondria, and the characteristic rod-like appearance of this organelle. The subcellular localization of the mitochondria is very similar to that observed in electron microscopy studies of these same cells (R. W. Craig, personal communication), and is similar to that observed when TMRE was used to monitor the mitochondrial membrane potential (Drummond & Fay, 1996).

Simultaneous measurement of cytoplasmic and mitochondrial $[Ca^{2+}]$ during depolarizing stimulation

The resting $[Ca^{2+}]_i$ in these smooth muscle cells, as measured with fura-2, was 119 ± 8 nM. During a 5 s train of depolarizing pulses (−110 to +10 mV, 250 ms, 2 Hz), $[Ca^{2+}]_i$ increased to 784 ± 87 nM ($n = 9$; Fig. 2*A*). The total

time to peak for the increase in $[Ca^{2+}]_i$ was $4{\cdot}1 \pm 0{\cdot}2$ s and the half-time ($t_{½}$) for recovery following the end of the depolarizing train was $4{\cdot}3 \pm 0{\cdot}2$ s (Fig. 2*C*). The same stimulus also increased mitochondrial $[Ca^{2+}]$ as indicated by the $49 \pm 7\%$ increase in rhod-2 fluorescence. When the cytoplasmic and mitochondrial $[Ca^{2+}]$ records from the same cell are viewed on an expanded time scale, it can clearly be seen that $[Ca^{2+}]_i$ increased faster than $[Ca^{2+}]_m$, and that $[Ca^{2+}]_m$ continues to increase even after $[Ca^{2+}]_i$ has reached a plateau (Fig. 2*B*). The $t_{½}$ to peak for the increase in rhod-2 fluorescence was $2{\cdot}6 \pm 0{\cdot}3$ s, which was significantly slower than the $t_{½}$ to peak for the increase in $[Ca^{2+}]_i$ (720 ± 50 ms) ($P < 0{\cdot}003$). The $t_{½}$ for recovery of rhod-2 fluorescence to its baseline value was $46{\cdot}7 \pm 11{\cdot}3$ s which was an order of magnitude longer than the $t_{½}$ for $[Ca^{2+}]_i$ recovery ($4{\cdot}3 \pm 0{\cdot}2$ s; $P < 0{\cdot}006$; Fig. 2*C*).

A consistent finding, which can be seen in the expanded time scale of the $[Ca^{2+}]_m$ record (Fig. 2*B*), is that while mitochondrial Ca^{2+} uptake begins immediately following initiation of the depolarizing stimulus there is an apparent slowing down of the rate of Ca^{2+} uptake soon afterwards. This is followed by a secondary increase in the rate of mitochondrial Ca^{2+} uptake (indicated by the arrow in Fig. 2*B*). The secondary increase in the rate of mitochondrial Ca^{2+} uptake occurred $2{\cdot}9 \pm 0{\cdot}9$ s after the beginning of the depolarizing train. Both phases of the increase in $[Ca^{2+}]_m$ were abolished by FCCP (1 μM; $n = 4$), indicating that the first phase was not due to contamination from residual cytoplasmic rhod-2.

Simultaneous measurement of cytoplasmic and mitochondrial $[Ca^{2+}]$ during caffeine-induced release of Ca^{2+} from the SR

Caffeine is capable of releasing Ca^{2+} from the SR in smooth muscle, by increasing the sensitivity of the ryanodine receptor to Ca^{2+}, thereby promoting Ca^{2+}-induced Ca^{2+} release. During a 5 s application of caffeine (20 mM), applied from a pressure ejection pipette positioned close to the cell,

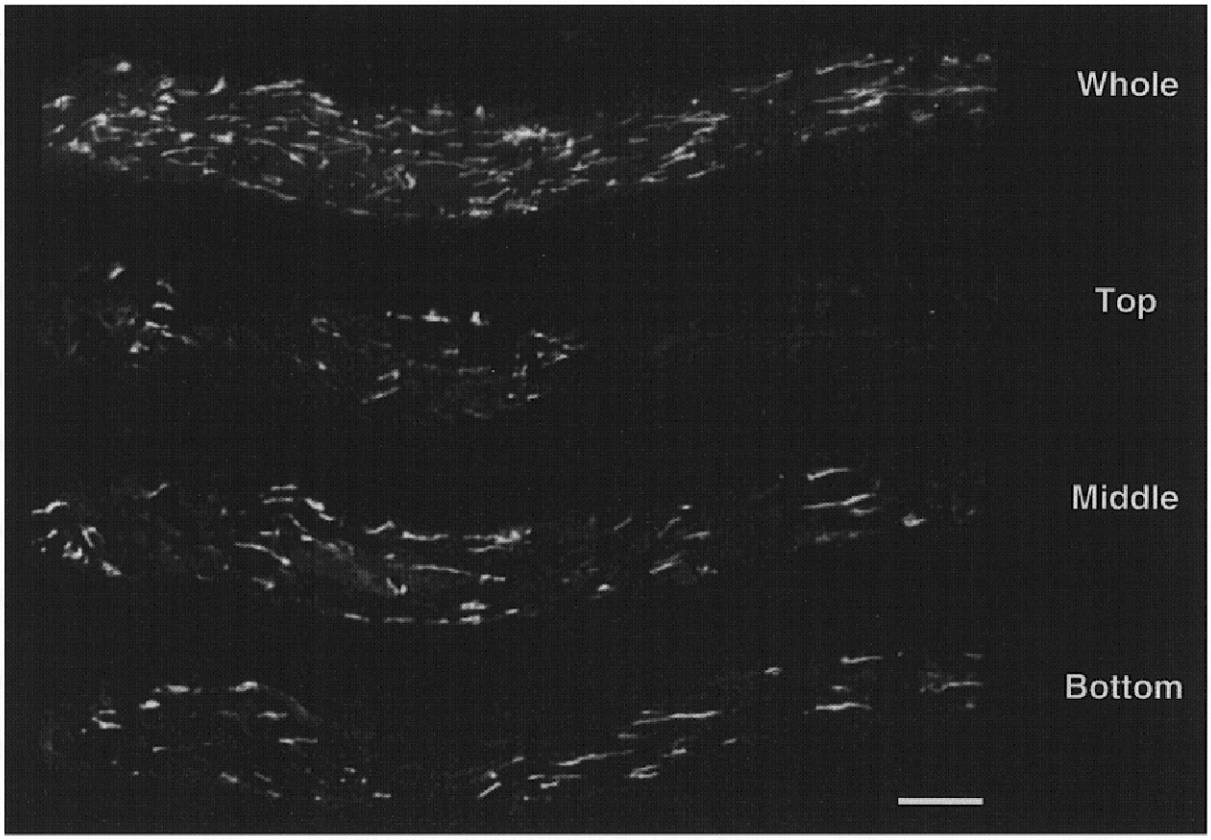

Figure 1. Mitochondria in a *Bufo marinus* gastric myocyte labelled with rhod-2

Smooth muscle cells were loaded with 1–1·5 μM rhod-2 AM for 1 h. The positive charge on rhod-2 AM results in significant accumulation of this indicator within the mitochondrial matrix. Following hydrolysis of the acetoxymethylester group, rhod-2 becomes trapped in the mitochondrial matrix. The image showing the whole cell reflects a maximum intensity projection of the entire through-focus data set. The images labelled top, middle and bottom reflect 0·25 μm optical sections from the respective locations within the cell. The cell was imaged using a digital imaging microscope (Moore *et al.* 1993) and processed using a constrained deconvolution algorithm (Carrington *et al.* 1990). Scale bar represents 10 μm.

$[Ca^{2+}]_i$ increased from a resting level of 113 ± 24 nM to 523 ± 90 nM ($n = 6$; Fig. 3*A*). The time to peak for the increase in $[Ca^{2+}]_i$ was $2{\cdot}1 \pm 0{\cdot}3$ s, and the $t_{½}$ for recovery was $4{\cdot}8 \pm 0{\cdot}4$ s. Stimulation with caffeine also produced an increase in rhod-2 fluorescence, indicative of an increase in $[Ca^{2+}]_m$. In contrast to the results shown in Fig. 2*B*, $[Ca^{2+}]_m$ increased with a similar time course to the increase in $[Ca^{2+}]_i$ during stimulation with caffeine (Fig. 3*B*). The rhod-2 fluorescence increased by $51 \pm 18\%$ with a $t_{½}$ to peak of $1{\cdot}0 \pm 0{\cdot}2$ s (Fig. 3*A* and *C*) and the $t_{½}$ for recovery was $20{\cdot}9 \pm 6{\cdot}6$ s (Fig. 3*C*). The $t_{½}$ to peak for the increase in $[Ca^{2+}]_m$ was not significantly different from that of the cytoplasm ($1{\cdot}1 \pm 0{\cdot}3$ s). As with depolarizing stimulation, $[Ca^{2+}]_m$ recovery back to baseline values was consistently slower than the recovery of $[Ca^{2+}]_i$.

Subcellular imaging of the increase in mitochondrial $[Ca^{2+}]$ during depolarizing stimulation

One advantage of using rhod-2 to monitor $[Ca^{2+}]_m$, as opposed to the use of mitochondrially targeted aequorin (Rizzuto *et al.* 1992), is the ability to resolve changes in $[Ca^{2+}]$ within individual mitochondria *in situ* (but see Rutter *et al.* 1996). Thus, we used rhod-2 in combination

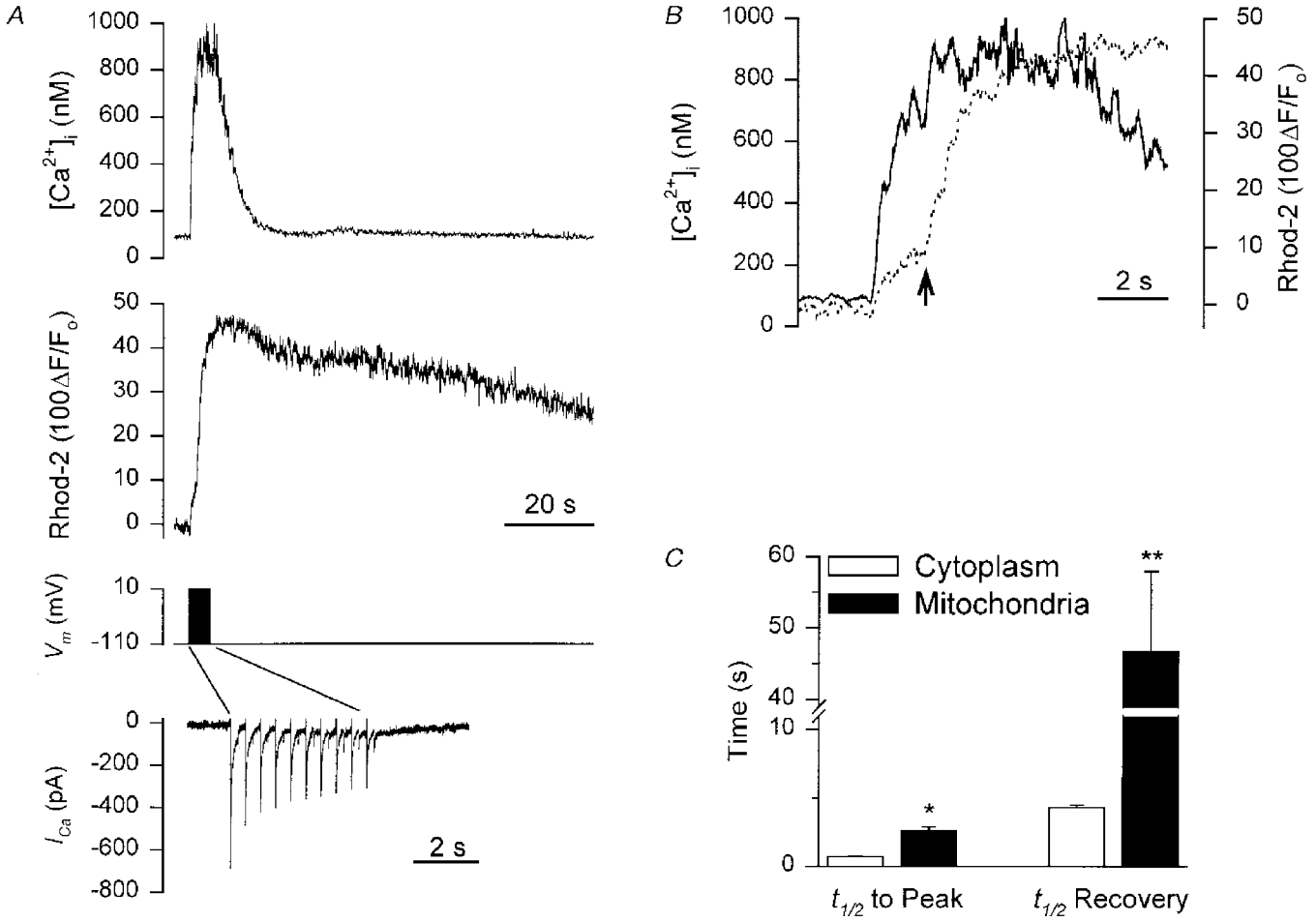

Figure 2. Simultaneous measurement of cytoplasmic and mitochondrial $[Ca^{2+}]$ during depolarizing stimulation

Smooth muscle cells were loaded with 1–1·5 μM rhod-2 AM for 1 h to enable monitoring of $[Ca^{2+}]_m$ and fura-2 was included in the patch pipette to allow simultaneous monitoring of $[Ca^{2+}]_i$. *A*, representative recording showing: top trace, the $[Ca^{2+}]_i$ transient during a 5 s train of depolarizing pulses (from −110 to +10 mV, 250 ms duration, 2 Hz); middle trace, the corresponding change in $[Ca^{2+}]_m$ during the depolarizing train; bottom traces show the membrane potential and the membrane current, during the depolarizing train. Note the change in time scale for the membrane current. *B*, overlay of $[Ca^{2+}]_i$ and $[Ca^{2+}]_m$ records from *A* re-plotted on an expanded time scale (continuous line, $[Ca^{2+}]_i$; dashed line, $[Ca^{2+}]_m$). Arrow indicates the secondary increase in the rate of mitochondrial Ca^{2+} uptake which occurs approximately 2 s after initiating the depolarizing train. *C*, summary data of the half-time ($t_{½}$) to peak for the increase in $[Ca^{2+}]_i$ and $[Ca^{2+}]_m$ and also the $t_{½}$ for the recovery of $[Ca^{2+}]_i$ and $[Ca^{2+}]_m$ ($n = 9$). * $P < 0{\cdot}003$, ** $P < 0{\cdot}006$.

with a high-speed digital imaging microscope to examine whether there were any marked differences in the responses of individual mitochondria to depolarizing stimulation, or following release of Ca^{2+} from the SR. During a 5 s train of depolarizing pulses (−110 to +10 mV, 250 ms, 2 Hz), which as indicated earlier would be expected to increase $[Ca^{2+}]_i$ to ~1 μM, there was a significant increase in mitochondrial rhod-2 fluorescence, indicative of mitochondrial Ca^{2+} uptake. Shown in pseudocolour in Fig. 4 is the change in rhod-2 fluorescence for individual mitochondria, within a 1·5 μm optical section from the through-focus set. The images represent the maximum intensity projection of three adjacent optical sections, which were near the central focus plane. Through-focus sets were acquired every 1 s during the depolarizing train, commencing 0·5 s after the start of the stimulus.

The high-speed digital imaging microscope provides the opportunity to examine the Ca^{2+} uptake properties of individual mitochondria, within specific cellular locations. In Fig. 4*D*, the rhod-2 fluorescence for six individual mitochondria, selected from within the 1·5 μm optical section, is shown. Each mitochondrion is represented by its pixel of maximum intensity. Three of the mitochondria were located close to the plasma membrane (open symbols), and the other three were located in the centre of the cell (closed symbols). Two of the six mitochondria showed no increase, or only a small (~10%) increase in rhod-2 fluorescence in

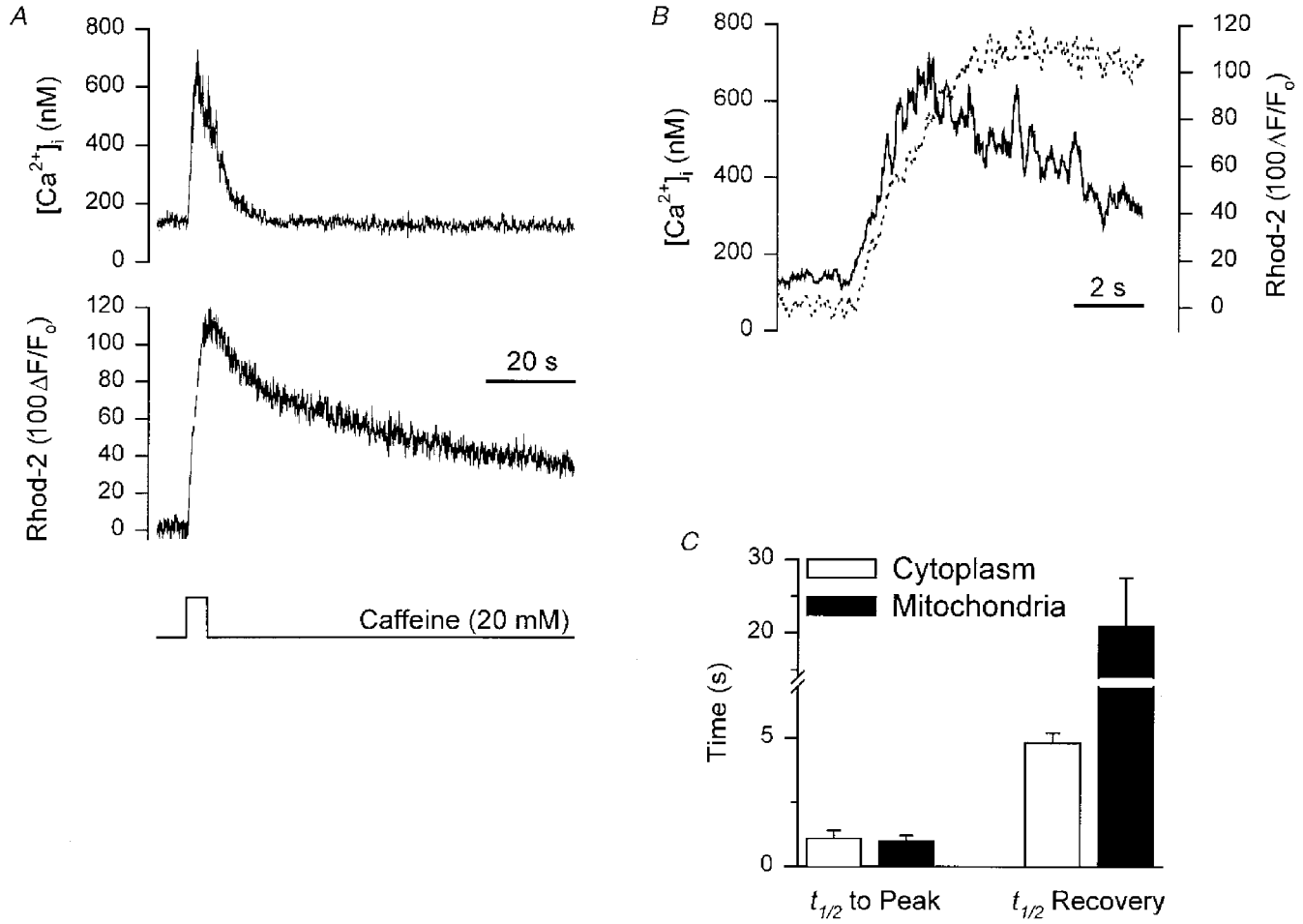

Figure 3. Simultaneous measurement of cytoplasmic and mitochondrial $[Ca^{2+}]$ during caffeine-induced release of Ca^{2+} from the SR

Smooth muscle cells were loaded with 1–1·5 μM rhod-2 AM for 1 h to enable monitoring of $[Ca^{2+}]_m$ and fura-2 was included in the patch pipette to allow simultaneous monitoring of $[Ca^{2+}]_i$. *A*, representative recording showing: top trace, the $[Ca^{2+}]_i$ transient in response to a 5 s application of caffeine (20 mM); middle trace, the corresponding change in $[Ca^{2+}]_m$ during the stimulation with caffeine; bottom trace the period of caffeine application. *B*, overlay of $[Ca^{2+}]_i$ and $[Ca^{2+}]_m$ records from *A* re-plotted on an expanded time scale (continuous line, $[Ca^{2+}]_i$; dashed line, $[Ca^{2+}]_m$). *C*, summary data of the $t_{½}$ to peak for the increase in $[Ca^{2+}]_i$ and $[Ca^{2+}]_m$ and also the $t_{½}$ for the recovery of $[Ca^{2+}]_i$ and $[Ca^{2+}]_m$ ($n = 6$).

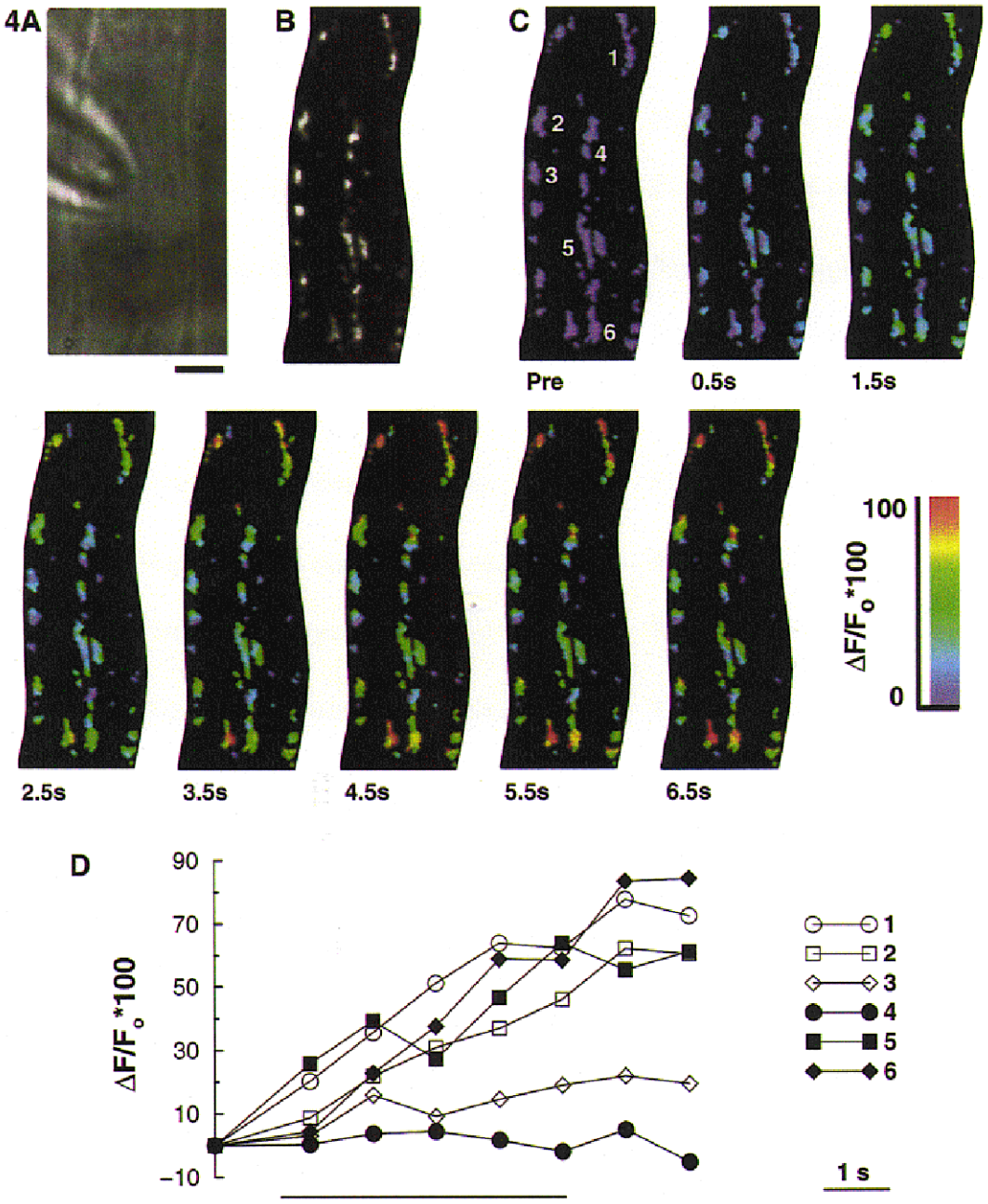

Figure 4. Subcellular imaging of the increase in mitochondrial [Ca^{2+}] during depolarizing stimulation

Smooth muscle cells were loaded with 1–1·5 μM rhod-2 AM for 1 h to enable monitoring of $[Ca^{2+}]_m$. *A*, bright field image of a *Bufo marinus* gastric smooth muscle cell from which the subsequent fluorescence images in this figure were obtained. Scale bar represents 5 μm and is applicable to all the images shown. *B*, grey scale image of rhod-2 labelled mitochondria. The image represents a 1·5 μm thick optical section from the through-focus data set, which has been processed using a constrained deconvolution algorithm (Carrington *et al.* 1990). *C*, shown in pseudocolour is the increase in $[Ca^{2+}]_m$, for the same optical section as in *B*, during a 5 s depolarizing train (from −110 to +10 mV, 250 ms duration, 2 Hz). 'Pre' indicates the image acquired before applying the depolarizing train. Thereafter, through-focus image sets were acquired every 1 s for the next 10 s, commencing 0·5 s after the beginning of the depolarizing train. *D*, rhod-2 fluorescence for the six individual mitochondria numbered in the first image of *C*, selected from regions close to the membrane (open symbols) or more centrally located (filled symbols), in the 3-D data set. Each mitochondrion is represented by the fluorescence of its brightest pixel, and was normalized to the pre-stimulation value. The bar beneath the graph indicates the period of depolarizing stimulation.

response to the depolarizing stimulus. At the end of the depolarizing train, the mitochondrial rhod-2 fluorescence had increased by 53 ± 4 % ($n = 7$ cells, 159 mitochondria).

Since the uptake of Ca^{2+} into the mitochondria is dependent upon the potential gradient across the inner membrane, collapsing this gradient should prevent mitochondria from taking up Ca^{2+}, thereby preventing the increase in rhod-2 fluorescence. When cells were exposed to the protonophore carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP; 1 μM), for 2–3 min before delivering the train of

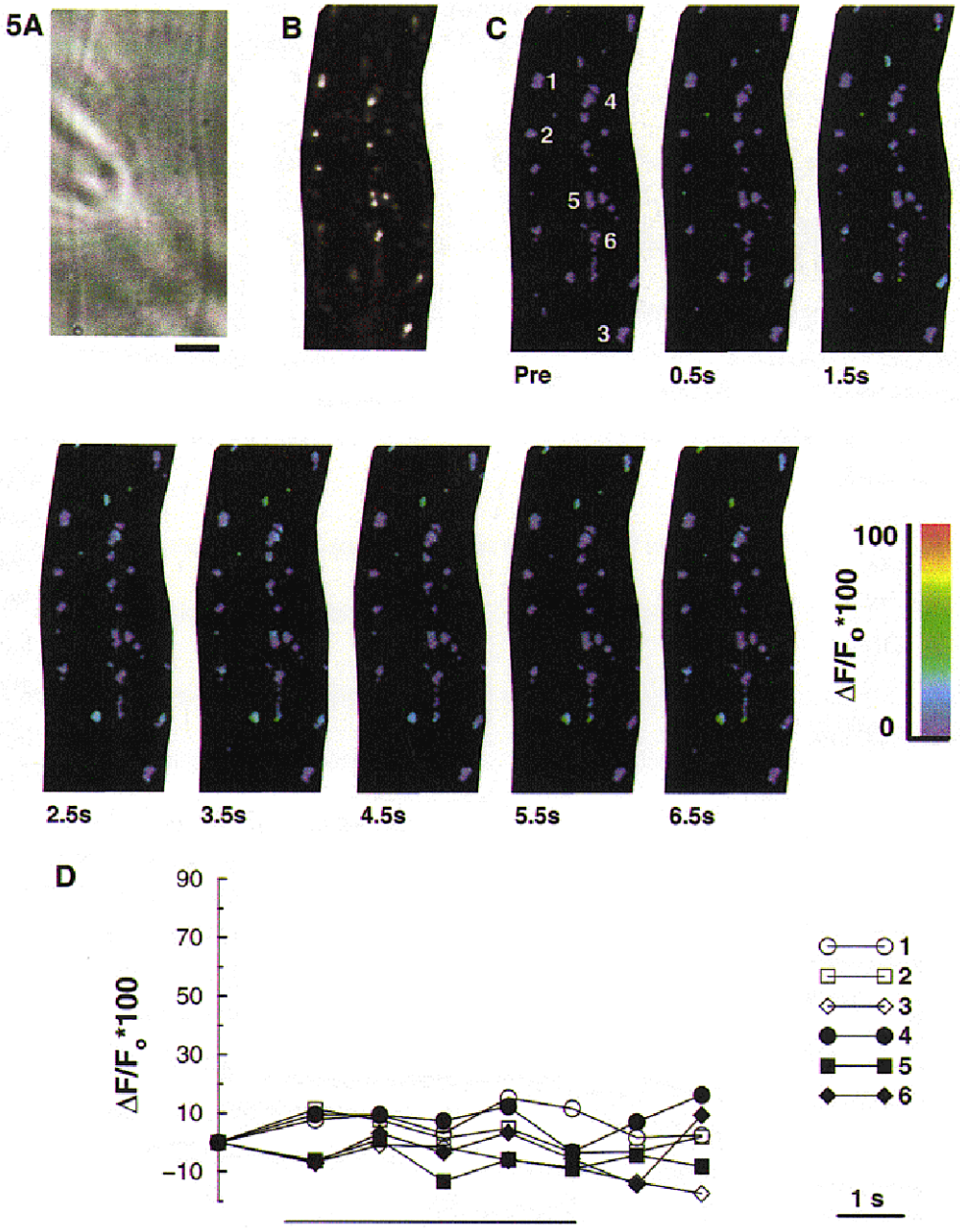

Figure 5. FCCP blocks the increase in mitochondrial [Ca^{2+}] during depolarizing stimulation

Smooth muscle cells were loaded with 1–1·5 μM rhod-2 AM for 1 h to enable monitoring of $[Ca^{2+}]_m$. *A*, bright field image of a *Bufo marinus* gastric smooth muscle cell from which the subsequent fluorescence images in this figure were obtained. Scale bar represents 5 μm and is applicable to all the images shown. *B*, grey scale image of rhod-2 labelled mitochondria. This image represents a 1·5 μm thick optical section of the through-focus data set. FCCP (1 μM) was applied to the cell approximately 3 min prior to delivering the depolarizing train. *C*, pseudocolour images of the same optical section as shown in *B* during a 5 s depolarizing train (from −110 to +10 mV, 250 ms duration, 2 Hz). 'Pre' indicates the image acquired before applying the depolarizing train. *D*, rhod-2 fluorescence for the six individual mitochondria numbered in the first image of *C*, selected from regions close to the membrane (open symbols) or more centrally located (filled symbols). Each mitochondrion is represented by the fluorescence of its brightest pixel, and was normalized to the pre-stimulation value. The bar beneath the graph indicates the period of depolarizing stimulation.

depolarizations, the increase in mitochondrial fluorescence was abolished (Fig. 5). In Fig. 5*D*, the rhod-2 fluorescence for six individual mitochondria which are located either close to the membrane (open symbols) or in the centre of the cell (closed symbols) is shown. We have previously shown that FCCP did not affect the magnitude of the $[Ca^{2+}]_i$ transient produced by depolarizing stimulation; however, it significantly slowed recovery of $[Ca^{2+}]_i$ back to its resting level (Drummond & Fay, 1996).

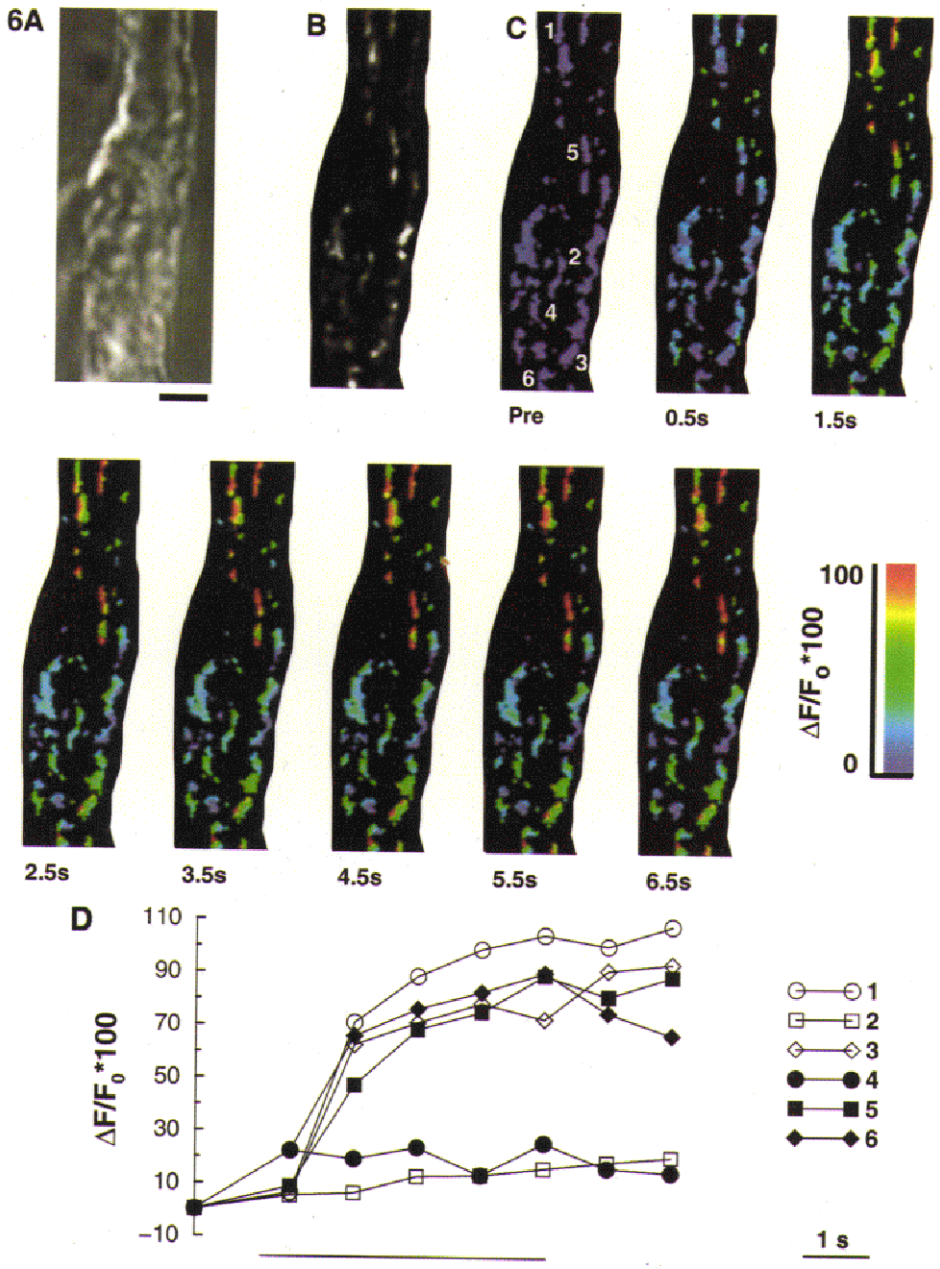

Figure 6. Subcellular imaging of the increase in mitochondrial $[Ca^{2+}]$ during caffeine-induced release of Ca^{2+} from the SR

Smooth muscle cells were loaded with 1–1·5 μM rhod-2 AM for 1 h to enable monitoring of $[Ca^{2+}]_m$. *A*, bright field image of a *Bufo marinus* gastric smooth muscle cell from which the subsequent fluorescence images in this figure were obtained. Scale bar represents 5 μm and is applicable to all the images shown. *B*, grey scale image of rhod-2 labelled mitochondria. This image represents a 1·5 μm thick optical section of the through-focus data set. *C*, pseudocolour images of the same optical section as shown in *B* during a 5 s application of caffeine (20 mM). 'Pre' indicates the image acquired before application of caffeine. *D*, rhod-2 fluorescence for the six individual mitochondria numbered in the first image of *C*, selected from regions close to the membrane (open symbols) or more centrally located (filled symbols). Each mitochondrion is represented by the fluorescence of its brightest pixel, and was normalized to the pre-stimulation value. The bar beneath the graph indicates the period of caffeine application.

Subcellular imaging of the increase in mitochondrial $[Ca^{2+}]$ during caffeine-induced release of Ca^{2+} from the SR

Application of caffeine to these smooth muscle cells typically produces a transient increase in $[Ca^{2+}]_i$, to approximately 500–750 nM, which normally returns to baseline values within 10–15 s (see above). During a 5 s application of caffeine (20 mM), which was applied from a pressure ejection pipette positioned close to the cell, mitochondrial rhod-2 fluorescence increased, which is indicative of mitochondrial Ca^{2+} uptake. Shown in pseudocolour in Fig. 6 is the change in rhod-2 fluorescence for individual mitochondria within a 1·5 μm optical section from the through-focus set. The images represent the maximum intensity projection of three adjacent optical sections, which were near the central focus plane. Through-focus sets were acquired every 1 s during the application of caffeine, commencing 0·5 s after the start of the stimulus.

In Fig. 6*D*, the rhod-2 fluorescence for six individual mitochondria, selected from within the 1·5 μm optical section, is shown. Each mitochondrion is represented by its pixel of maximum intensity. Three of the mitochondria were located close to the plasma membrane (open symbols), and the other three were located in the centre of the cell (closed symbols). At the end of the 5 s caffeine application, the mitochondrial rhod-2 fluorescence had increased by 55 ± 4 % ($n = 5$ cells, 115 mitochondria).

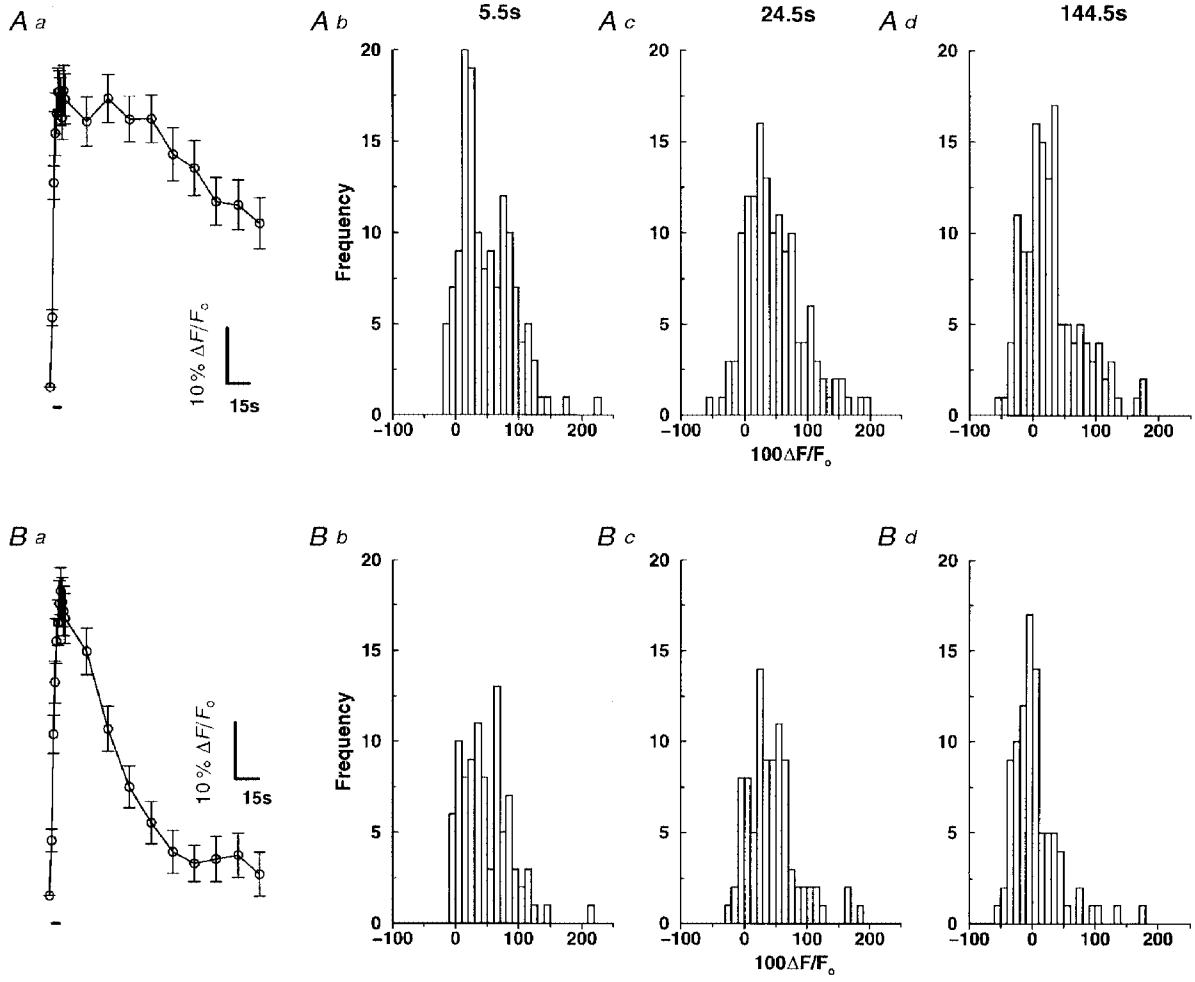

Figure 7. Time course of depolarization- and caffeine-induced change in rhod-2 fluorescence

Smooth muscle cells were loaded with 1–1·5 μM rhod-2 AM for 1 h to enable monitoring of $[Ca^{2+}]_m$. *Aa*, time course of the change in mitochondrial rhod-2 fluorescence during a brief train of depolarizations. Approximately 20–30 mitochondria were selected in each cell and the brightest pixel was then followed through time ($n = 7$ cells, 159 mitochondria). The bar beneath the graph represents the period of depolarizing stimulation. *Ab–d*, histograms of the change in rhod-2 fluorescence at $t = 5·5$, 24·5 and 144·5 s for the depolarizing stimulus. *Ba*, time course of the change in mitochondrial rhod-2 fluorescence during a 5 s caffeine application. Approximately 20–30 mitochondria were selected in each cell and the brightest pixel was then followed through time ($n = 5$ cells, 115 mitochondria). The bar beneath the graph represents the period of caffeine stimulation. *Bb–d*, histograms of the change in rhod-2 fluorescence at $t = 5·5$, 24·5 and 144·5 s for stimulation with caffeine.

Time course of the depolarization- and caffeine-induced change in mitochondrial $[Ca^{2+}]$

Figure 7*A* shows the time course of the depolarization-induced change in rhod-2 fluorescence, determined from the images of 159 individual mitochondria *in situ* ($n = 7$ cells). At the end of the depolarizing train, rhod-2 fluorescence had increased by $53 \pm 4\%$, with approximately 80% of the mitochondria showing a $\geqslant 10\%$ increase in rhod-2 fluorescence. A further 19·5 s after the end of the stimulus, when $[Ca^{2+}]_i$ would normally be back at its resting level, mitochondrial rhod-2 fluorescence remained elevated by $47 \pm 4\%$. Even at 139·5 s following the end of the depolarizing stimulus, mitochondrial rhod-2 fluorescence was still elevated by $29 \pm 5\%$. Also shown in Fig. 7*A* are frequency histograms of the rhod-2 fluorescence change for individual mitochondria at $t = 5{\cdot}5$ s (Fig. 7*Ab*) which is 0·5 s after the end of the depolarizing train; at $t = 24{\cdot}5$ s (Fig. 7*Ac*) which is 19·5 s after the end of the stimulus; and at $t = 144{\cdot}5$ s (Fig. 7*Ad*) which is at the end of the recording period.

Figure 7*B* shows the time course of caffeine-induced change in rhod-2 fluorescence, determined from the images of 115 individual mitochondria *in situ* ($n = 5$). At the end of the 5 s application of caffeine, mitochondrial fluorescence had increased by $55 \pm 4\%$, with approximately 82% of the

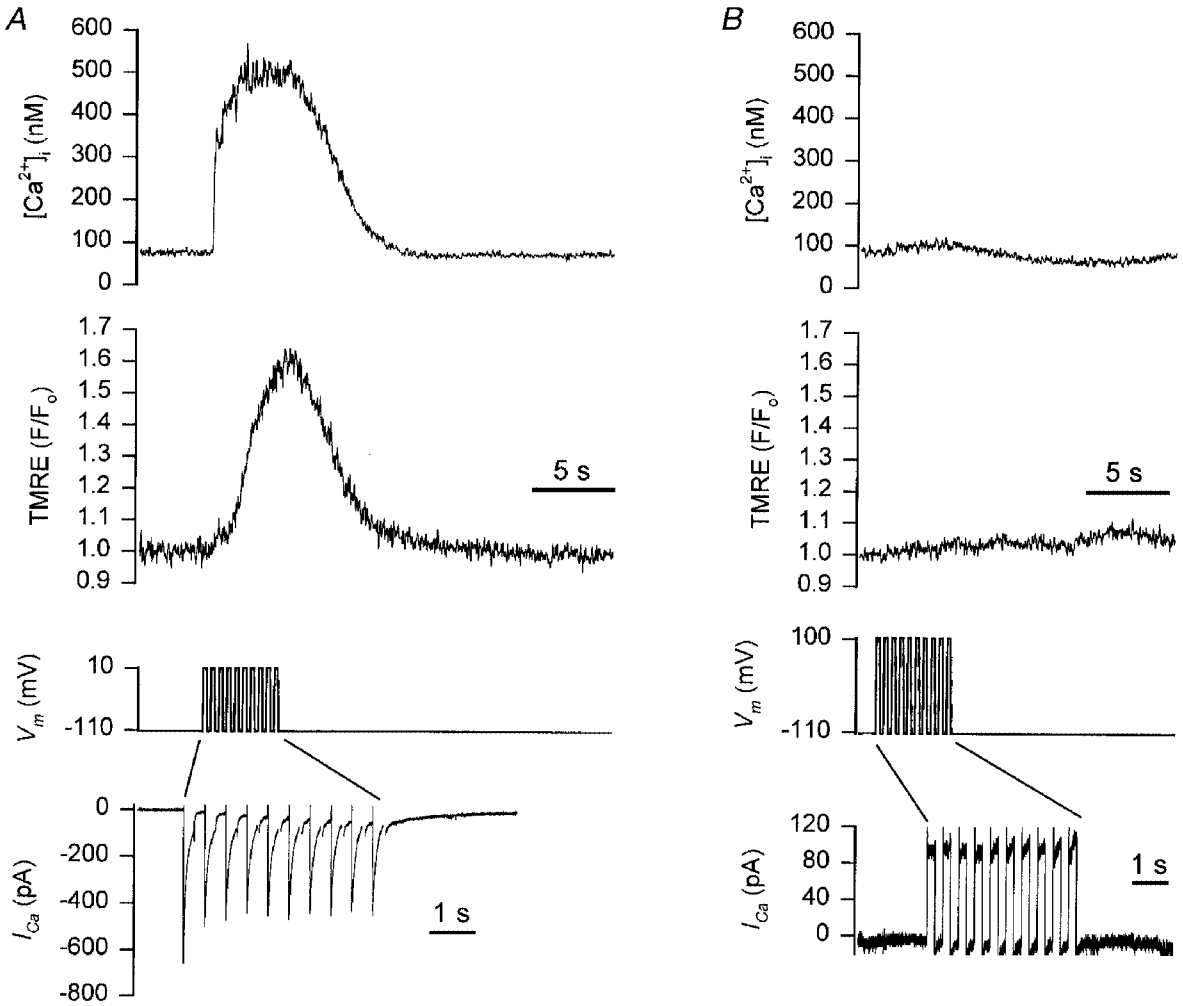

Figure 8. Simultaneous measurement of cytoplasmic $[Ca^{2+}]$ and mitochondrial membrane potential during depolarizing stimulation

Smooth muscle cells were loaded with 100 nM TMRE for 10 min to enable monitoring of the mitochondrial membrane potential. Fura-2 was included in the patch pipette to allow simultaneous monitoring of $[Ca^{2+}]_i$. *A*, representative recording showing: top trace, the $[Ca^{2+}]_i$ transient during a 5 s train of depolarizing pulses (from −110 to +10 mV, 250 ms duration, 2 Hz); middle trace, the corresponding change in mitochondrial membrane potential. An increase in TMRE fluorescence signals depolarization of the mitochondrial membrane potential; bottom traces show the membrane potential and the membrane current, during the depolarizing train. Note the change in the time scale for the membrane current. *B*, the same cell as shown in *A*, with the membrane potential in this sequence pulsed to +100 mV during the depolarizing train.

mitochondria showing a $\geqslant 10\%$ increase in rhod-2 fluorescence at this time. At 19·5 s post-caffeine application, when $[Ca^{2+}]_i$ would be back at baseline levels, mitochondrial rhod-2 fluorescence was still elevated by $30 \pm 4\%$. The mitochondrial rhod-2 fluorescence was restored to baseline values approximately 60 s after stimulation with caffeine. Figure 7*B* shows frequency histograms of the rhod-2 fluorescence change for individual mitochondria at $t = 5{\cdot}5$ s (Fig. 7*Bb*) which is 0·5 s after the end of the period of caffeine application; at $t = 24{\cdot}5$ s (Fig. 7*Bc*) which is 19·5 s after the end of the stimulus; and at $t = 144{\cdot}5$ s (Fig. 7*Bd*) which is at the end of the recording period.

Simultaneous measurement of cytoplasmic $[Ca^{2+}]$ and mitochondrial membrane potential

The lipophilic cation TMRE was used to monitor the mitochondrial membrane potential during depolarizing stimulation, while simultaneously monitoring $[Ca^{2+}]_i$ with fura-2. During a train of depolarizing pulses (−110 to +10 mV, 250 ms, 2 Hz), $[Ca^{2+}]_i$ increased from 126 ± 11 nM to 637 ± 66 nM with a $t_{½}$ to peak of 170 ± 20 ms ($n = 6$). The $t_{½}$ for recovery of $[Ca^{2+}]_i$ to baseline values following the end of the depolarizing train was $3{\cdot}9 \pm 0{\cdot}4$ s. Simultaneous measurement of TMRE fluorescence indicated a depolarization of the mitochondrial membrane potential. This is manifest as an increase in TMRE fluorescence, as depolarization of the mitochondria membrane potential leads to redistribution of TMRE into the cytoplasm, and relief of fluorescence quenching (Duchen *et al.* 1998; Boitier *et al.* 1999). Confirmation of this effect was provided by application of FCCP (1 μM) which caused a $132 \pm 10\%$ increase in TMRE fluorescence. During the depolarizing train TMRE fluorescence increased by $48 \pm 11\%$, with a $t_{½}$ to a peak of $1{\cdot}7 \pm 0{\cdot}3$ s (Fig. 8*A*). The TMRE fluorescence recovered to baseline values with a $t_{½}$ of $4{\cdot}5 \pm 1{\cdot}3$ s.

Two further observations support the notion that the change in mitochondrial membrane potential results from the influx of Ca^{2+} into the matrix and consequent membrane depolarization. Firstly, when 10 mM BAPTA was included in the patch pipette, the increase in $[Ca^{2+}]_i$ normally seen during the depolarizing train was prevented and there was no change in TMRE fluorescence (data not shown). Secondly, when the membrane potential was stepped to +100 mV during the depolarizing train, so as to prevent Ca^{2+} entry, there was no increase in $[Ca^{2+}]_i$ and also no increase in TMRE fluorescence (Fig. 8*B*). It should be noted that while $[Ca^{2+}]_m$ required tens of seconds to recover to its baseline value (Fig. 2*C*), the mitochondrial membrane potential recovered much more rapidly following the train of depolarizing pulses.

DISCUSSION

Over the past several years, there have been an increasing number of studies indicating mitochondrial involvement in $[Ca^{2+}]_i$ regulation in a variety of cell types including: neurons (Duchen *et al.* 1990; Thayer & Miller, 1990; Friel & Tsien, 1994); chromaffin cells (Herrington *et al.* 1996); cardiac myocytes (Bassani *et al.* 1992); and smooth muscle cells (Drummond & Fay, 1996). While many of these studies utilized pharmacological approaches to implicate mitochondrial involvement in $[Ca^{2+}]_i$ regulation, they have been substantiated recently by using techniques which allow direct monitoring of mitochondrial $[Ca^{2+}]$. While perhaps the most elegant approach utilized has been that of recombinant DNA technology to target the Ca^{2+}-sensitive photoprotein aequorin to the mitochondria (Rizzuto *et al.* 1992), there are some disadvantages associated with this technique. First, the cells being studied often have to be maintained in culture to enable transfection with the mitochondrially targeted aequorin. Thus, this approach cannot easily be applied to freshly dissociated cells. Second, aequorin is consumed upon binding Ca^{2+} and it is also sensitive to changes in the Mg^{2+} concentration. Third, using this approach, it is difficult to make measurements from single cells (but see Rutter *et al.* 1996); hence, resolution at the level of individual mitochondria is not possible.

An alternative methodology to monitor $[Ca^{2+}]_m$, which has been used successfully by an increasing number of laboratories, is the Ca^{2+} sensitive fluorescent indicator rhod-2 (Mix *et al.* 1994; Sheu & Jou, 1994; Hajnöczky *et al.* 1995; Babcock *et al.* 1997; Drummond & Tuft, 1999). The acetoxymethyl (AM) ester of rhod-2 is the only cell-permeant Ca^{2+} indicator which has a net positive charge. This property readily promotes compartmentalization of rhod-2 AM into the mitochondrial matrix, most likely via mitochondrial membrane potential driven uptake when cells are exposed to the AM ester. Once inside the mitochondrial matrix, esterases hydrolyse the AM group leaving the Ca^{2+}-sensitive form of rhod-2 trapped by virtue of its membrane impermeability. One major advantage of the approach of using rhod-2 to monitor $[Ca^{2+}]_m$, as opposed to aequorin, is the ability to make measurements of $[Ca^{2+}]_m$ not only at the single cell level, but also from individual mitochondria *in situ*.

Kinetics of mitochondrial $[Ca^{2+}]$ increase relative to the increase in cytoplasmic $[Ca^{2+}]$

By simultaneously monitoring cytoplasmic and mitochondrial $[Ca^{2+}]$ with high temporal resolution, it is possible to obtain kinetic information pertaining to the $[Ca^{2+}]$ changes within these two compartments. Thus, during a brief train of depolarizing pulses, which promotes Ca^{2+} influx from the extracellular medium, there was a rapid increase in both $[Ca^{2+}]_i$ and $[Ca^{2+}]_m$. When the $t_{½}$ to peak for the increase in $[Ca^{2+}]_i$ was compared with that of the increase in $[Ca^{2+}]_m$, it was found that the $[Ca^{2+}]$ increased more rapidly in the cytoplasm than in the mitochondria. When cells were stimulated with caffeine, which promotes release of Ca^{2+} from the SR via the ryanodine receptor, there was also an increase in both $[Ca^{2+}]_i$ and $[Ca^{2+}]_m$. However, unlike the depolarizing stimuli, there was no significant difference between the $t_{½}$ to peak for the increase in $[Ca^{2+}]_i$ or $[Ca^{2+}]_m$. Although depolarization and caffeine stimulation produced increases

in rhod-2 fluorescence with different time courses, the magnitude of the increase was the same for both.

Since the train of depolarizations produced a significantly higher increase in $[Ca^{2+}]_i$ than that produced by caffeine stimulation, bulk cytoplasmic $[Ca^{2+}]$ does not appear to be a good predictor of either the magnitude or kinetics of mitochondrial Ca^{2+} uptake in these cells. Rather, the differences in mitochondrial Ca^{2+} uptake may relate to the subcellular location of mitochondria relative to the sites of Ca^{2+} influx or Ca^{2+} release (Rizzuto *et al.* 1993; Lawrie *et al.* 1996; Rizzuto *et al.* 1998). In this respect, it is known from electron microscopy studies in smooth muscle that mitochondria are closely apposed to the SR, with the distance between these two organelles being approximately 20 nm (Nixon *et al.* 1994). Consequently, for a brief period of time after the SR Ca^{2+} release channels have been activated, mitochondria could be exposed to much higher Ca^{2+} concentrations than that observed in the bulk cytoplasm (Rizzuto *et al.* 1993; Rizzuto *et al.* 1998), leading to rapid accumulation of Ca^{2+} by the mitochondria. Clearly, further studies would have to be carried out using this particular cell type to determine the subcellular localization of mitochondria relative to the SR and plasma membrane, in order to substantiate this hypothesis.

Differences in the magnitude and kinetics of mitochondrial Ca^{2+} uptake have been observed in other cell types, depending upon the source of Ca^{2+} mobilized by the excitatory stimulus. Specifically, in endothelial cells, stimulation by extracellular application of ATP causes a long lasting increase in $[Ca^{2+}]_m$ that largely depends on Ca^{2+} influx. Release of stored Ca^{2+} is ineffective at increasing $[Ca^{2+}]_m$ in these endothelial cells (Lawrie *et al.* 1996). This is in contrast to studies in HeLa cells where Ca^{2+} release from intracellular stores was found to be more effective at increasing $[Ca^{2+}]_m$, compared with Ca^{2+} influx following depletion of the intracellular Ca^{2+} stores (Rizzuto *et al.* 1993). For the smooth muscle cells used in the present study, both depolarization-induced stimulation of Ca^{2+} influx and caffeine-induced release of Ca^{2+} from the SR were effective at increasing rhod-2 fluorescence, indicative of mitochondrial Ca^{2+} uptake. As mentioned earlier, however, differences in the kinetics of mitochondrial Ca^{2+} uptake were apparent, depending upon the mode of stimulation.

A consistent finding observed during depolarizing stimulation was that while Ca^{2+} uptake into mitochondria occurred immediately upon initiating the depolarizing train, soon afterwards it appeared to slow down. At approximately 2 s into the depolarizing train, however, there was a secondary increase in the rate of mitochondrial Ca^{2+} uptake. Since both phases of the response were abolished by FCCP, it is unlikely that the first phase is due to contamination of the fluorescence signal by cytoplasmic rhod-2. Such discrete phases of mitochondrial Ca^{2+} uptake were not observed during stimulation of these cells with caffeine. Although it is not clear what is responsible for the different phases of mitochondrial Ca^{2+} uptake, similar findings have been reported for endothelial cells (Donnadieu & Bourguignon, 1996) and cultured smooth muscle cells (Monteith & Blaustein, 1999). It could be that the initial phase of mitochondrial uptake reflects the activity of a previously described rapid mode of Ca^{2+} uptake (Sparagna *et al.* 1995), and that the secondary increase in mitochondrial Ca^{2+} uptake represents an allosteric activation of the uniporter by Ca^{2+} (Gunter & Pfeiffer, 1990). It is also possible that the initial phase reflects Ca^{2+} uptake by relatively few mitochondria, responding to local domains of high $[Ca^{2+}]$. It is interesting to note that a previous study in these same smooth muscle cells, describing a Ca^{2+}–calmodulin-dependent protein kinase II-mediated acceleration of Ca^{2+} removal processes, determined that the upregulation developed with a latency of 1–2 s (McGeown *et al.* 1996, 1998).

Imaging changes in mitochondrial $[Ca^{2+}]$ *in situ*

The use of rhod-2 as an indicator of $[Ca^{2+}]_m$ enables monitoring of matrix $[Ca^{2+}]$ at the level of individual mitochondria. Thus, when mitochondria were imaged during a train of depolarizations, or stimulation with caffeine, an increase in $[Ca^{2+}]$ within individual mitochondria could be observed. At the end of the 5 s stimulus, approximately 44 and 38 % of the mitochondria had shown a $\geqslant 50$ % increase in rhod-2 fluorescence for depolarization and caffeine stimulation, respectively. This increase in $[Ca^{2+}]_m$ during the depolarizing train was abolished by FCCP, indicating that rhod-2 was reliably monitoring the matrix $[Ca^{2+}]$. When the frequency histograms, representing mitochondrial rhod-2 fluorescence at the end of the 5 s stimulus, were compared for both depolarizing and caffeine stimulation, they were found to be similar according to the Kolmogorov–Smirnov test. It is evident from the frequency histograms that some mitochondria show only a small (< 20 %) increase in rhod-2 fluorescence during either form of stimulation. Since Ca^{2+} uptake by mitochondria is dependent upon the mitochondrial membrane potential, one explanation why some mitochondria show only a small increase in $[Ca^{2+}]_m$ is that they are more depolarized and thus, less effective at taking up Ca^{2+}. To verify this possibility, it would be necessary to monitor mitochondrial membrane potential and $[Ca^{2+}]_m$ simultaneously. However, the similarity in fluorescence spectra for rhod-2 and TMRE limits our ability to use these two indicators simultaneously at the present time. It is also possible that the mitochondria showing small responses have normal membrane potential, and that the lack of Ca^{2+} uptake reflects exposure to radically different Ca^{2+} levels during stimulation. Further studies are required in order to determine the nature of the heterogeneous mitochondrial Ca^{2+} responses.

Recently it has been shown that focal Ca^{2+} release from the SR, commonly referred to as Ca^{2+} sparks (Cheng *et al.* 1993), can cause transient mitochondrial membrane depolarizations in rat cardiac myocytes (Duchen *et al.* 1998). We have also observed individual mitochondria, within *Bufo marinus* gastric smooth muscle cells, showing transient depolarizations in membrane potential under resting conditions (data not

shown). Since Ca^{2+} sparks have been observed in these cells (ZhuGe *et al.* 1999) a similar phenomenon may be occurring as that observed in cardiac myocytes (Duchen *et al.* 1998), which may explain why some mitochondria are less effective at taking up Ca^{2+}. When the time course of the change in $[Ca^{2+}]_m$ was compared for mitochondria in the periphery *versus* the centre of the cell, the responses were remarkably similar (see Figs 3 and 5). Thus, the difference between mitochondria which were observed to be effectively taking up Ca^{2+}, and those which were less effective, does not appear to be related to their subcellular localization.

Kinetics of mitochondrial efflux

It is not clear why the efflux of Ca^{2+} from the mitochondria is so slow, relative to the rate of removal of cytoplasmic Ca^{2+}. From Figs 2 and 3 it is evident that $[Ca^{2+}]_i$ returns to its baseline value considerably faster than the decline in rhod-2 fluorescence. This finding is consistent with several other studies in smooth muscle cells (Drummond & Tuft, 1999; McCarron & Muir, 1999; Monteith & Blaustein, 1999). The slow rate of mitochondrial Ca^{2+} efflux observed in smooth muscle cells is in marked contrast to studies in hepatocytes, where $[Ca^{2+}]_m$ was found to be restored to resting levels while $[Ca^{2+}]_i$ remained elevated (Hajnöczky *et al.* 1995). However, a recent study in astrocytes showed that $[Ca^{2+}]_m$ can take approximately 30 min to return to its baseline value following stimulation with extracellular application of ATP (Boitier *et al.* 1999). Thus, there appear to be significant differences in the kinetics of mitochondrial Ca^{2+} efflux, which possibly reflect specific cell type variations and which may be related to the characteristics of the cytoplasmic Ca^{2+} response typically seen in a given cell (see Hajnöczky *et al.* 1995).

The slow rate of mitochondrial Ca^{2+} efflux was also observed when rhod-2 was used to monitor the $[Ca^{2+}]_m$ of individual mitochondria *in situ*. Specifically, at 19·5 s after the depolarizing train or caffeine application, a $\geqslant 50\,\%$ increase in rhod-2 fluorescence was still evident in 81 and 34 % of the mitochondria for the respective stimuli. Studies on mitochondria isolated from smooth muscle have shown that, unlike most other cell types, they do not have a Na^+-dependent Ca^{2+} efflux mechanism (Crompton *et al.* 1978), which may account in part for the slow rate of efflux. In this respect a brief increase in $[Ca^{2+}]_i$ in smooth muscle could produce a more prolonged increase in $[Ca^{2+}]_m$, which may have important consequences for stimulation of oxidative phosphorylation (McCormack *et al.* 1990).

The present study has also shown that the recovery of $[Ca^{2+}]_m$ to resting values is quicker when the source of Ca^{2+} is the SR as opposed to the extracellular medium. Specifically, at the end of the recording period, a $\Delta F/F_o \geqslant 50\,\%$ was still evident in 26 % of the mitochondria following depolarizing stimulation, compared with 8 % of the mitochondria following stimulation with caffeine. In this regard we offer the following hypothesis. When Ca^{2+} release from the SR is stimulated, the mitochondria will take up Ca^{2+} and the SR will be depleted. If the mitochondria and SR are closely coupled in these cells, as suggested for other smooth muscle cells (Nixon *et al.* 1995), then efflux of Ca^{2+} from the mitochondria to a depleted SR may provide an efficient mechanism for restoring $[Ca^{2+}]_m$ to its resting value. In response to depolarizing stimulation, mitochondria will take up Ca^{2+} but the SR is not significantly depleted (Mlinar *et al.* 1995). Thus, efflux of Ca^{2+} from the mitochondria back to the SR under these conditions may not be as effective a mechanism. This notion is in agreement with a previous study in cardiac myocytes, showing that the mitochondria are capable of supplying Ca^{2+} which is utilized to replenish the SR (Bassani *et al.* 1993).

Effect of mitochondrial Ca^{2+} uptake on mitochondrial membrane potential

Since Ca^{2+} uptake into mitochondria during physiological stimulation has been shown to cause depolarization of the mitochondrial membrane potential (Loew *et al.* 1994) we were interested to determine whether a similar effect can be observed in smooth muscle. Indeed, Ca^{2+} influx during the depolarizing train caused a transient change in the mitochondrial membrane potential. Since the activity of the ATP synthetase is dependent upon the mitochondrial membrane potential, depolarization of the mitochondria would lead to a decrease in ATP production. Thus, it is important for the cell to maintain the mitochondrial membrane potential, but how this is achieved is unknown at the present time. Possibilities are that the electron transport chain is stimulated thereby re-establishing the mitochondrial membrane potential, or ATP is consumed by the ATP synthetase in order to pump protons across the mitochondrial membrane and maintain the potential.

It is interesting to note that whereas the change in mitochondrial membrane potential during the depolarizing train is somewhat transient in nature, with a time course very similar to the change in $[Ca^{2+}]_i$, Ca^{2+} in the mitochondria remains elevated for a considerably longer period of time. Since the mitochondrial membrane potential is required in order for the ATP synthetase to function, this may be the reason why the change in mitochondrial membrane potential is transient in nature. The more prolonged elevation in $[Ca^{2+}]_m$ could conceivably lead to a prolonged activation of oxidative phosphorylation, through the stimulatory effect of Ca^{2+} on several mitochondrial dehydrogenases as mentioned earlier.

The results of the present study further support and extend our previous findings that mitochondria in smooth muscle cells are capable of contributing to cytoplasmic Ca^{2+} removal, by taking up Ca^{2+} which has entered the cytoplasm following depolarizing stimulation (Drummond & Fay, 1996), or activation of SR Ca^{2+} release. Specifically, using the Ca^{2+}-sensitive fluorescent indicator rhod-2 we were able to observe changes in $[Ca^{2+}]_m$ not only at the single cell level, but also at the level of individual mitochondria *in situ*. Our findings are in agreement with

several other studies in smooth muscle, showing increases in $[Ca^{2+}]_m$ following the increase in $[Ca^{2+}]_i$ produced by depolarization of the plasma membrane and Ca^{2+} influx (McCarron & Muir, 1999), or agonist induced release of Ca^{2+} from the SR (Drummond & Tuft, 1999; Monteith & Blaustein, 1999).

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