THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 271, No. 18, Issue of May 3, pp. 10753–10759, 1996

Printed in U.S.A.

A Role for Calcium Influx in the Regulation of Mitochondrial Calcium in Endothelial Cells*

(Received for publication, September 25, 1995, and in revised form, February 26, 1996)

Alison M. Lawrie, Rosario Rizzuto‡, Tullio Pozzan‡, and Alec W. M. Simpson§

From the Department of Human Anatomy and Cell Biology, University of Liverpool, Ashton Street, Liverpool L69 3BX, United Kingdom and the ‡Department of Biomedical Sciences, University of Padova, Via Trieste 75, 35121 Padova, Italy

By using an endothelial cell line (ECV304), derived from human umbilical vein and transfected with recombinant aequorin targeted to the mitochondrial matrix, we find that stimulation with ATP evokes long lasting increases in mitochondrial Ca^{2+} ($[Ca^{2+}]_m$) that largely depend on Ca^{2+} influx. In these cells, the release of stored Ca^{2+} is inefficient at elevating $[Ca^{2+}]_m$. Consequently it appears that in ECV304 cells, bulk cytosolic Ca^{2+} ($[Ca^{2+}]_c$) is the main determinant of $[Ca^{2+}]_m$ changes.

In ECV304 cells <4% of mitochondria are within 700 nm of the endoplasmic reticulum as opposed to 65% in HeLa cells, whereas 14% are within 700 nm of the inner surface of the plasma membrane, compared with <6% in HeLa cells. Following Ca^{2+} depletion, readdition of extracellular Ca^{2+} evokes an increase in $[Ca^{2+}]_m$ but not in $[Ca^{2+}]_c$. Under these conditions, microdomains of high $[Ca^{2+}]_c$ may occur beneath the plasma membrane of ECV304 cells resulting in the preferential elevation of Ca^{2+} in mitochondria located in this region.

A model is discussed in which the localization of mitochondria with respect to Ca^{2+} sources is the main determinant of their *in situ* Ca^{2+} uptake kinetics. Thus, in any given cell type mitochondria may be localized to suit the energy and metabolic demands of their physiological actions.

It has long been known that stimulation of nonexcitable cells with phosphatidylinositol bisphosphate-coupled agonists leads to a rise in the concentration of cytosolic calcium ($[Ca^{2+}]_c$)[1] via the action of inositol 1,4,5-trisphosphate ($InsP_3$) on intracellular calcium (Ca^{2+}) stores and that this increase is important for the manifestation of many cellular processes (1). Agonist-evoked Ca^{2+} release is associated with rises in the concentration of calcium in the matrix of the mitochondria ($[Ca^{2+}]_m$) (2–4). High $[Ca^{2+}]_m$ has been shown to stimulate the activity of key metabolic enzymes (5, 6) resulting in raised energy production for the stimulated cell. Ca^{2+} enters the mitochondria through a uniporter, present in the inner membrane, and can be exported via the stimulation of H^+:Ca^{2+} and Na^+:Ca^{2+} antiporters thus creating a Ca^{2+} cycling system for the control of $[Ca^{2+}]_m$ (7–10).

Until recently the estimation of $[Ca^{2+}]_m$ changes *in vivo* was only possible by following the activation of the metabolic enzymes by Ca^{2+} (5, 6) or loading cells with high levels of a fluorescent indicator, followed by quenching of the cytosolic dye (11). Here, we have utilized recombinant Ca^{2+}-dependent luminescent photoprotein aequorin specifically targeted to the mitochondria (mtAEQ) (2, 12) to monitor $[Ca^{2+}]_m$ during agonist activation. In particular we are interested in the changes in $[Ca^{2+}]_m$ evoked by stimulation of an endothelial cell line with the physiological agonist ATP. For this purpose we have chosen a cell line, designated ECV304, that was spontaneously immortalized from a primary culture of human umbilical vein endothelial cells (13).

ATP is known to be a physiologically important stimulator of endothelial cells. Its activation of the cells results chiefly in the production of the vasodilators prostacyclin and endothelial-derived relaxing factor, now known to be nitric oxide (14), along with other factors important in the control of vascular tone (15). ATP binds plasma membrane receptors of the purinergic P_{2Y} classification.

Previous studies, including those on HeLa cells, have shown that the pattern of mitochondrial response to phosphatidylinositol bisphosphate-coupled agonists in nonexcitable cells is one of a rapid, large, and transient elevation in $[Ca^{2+}]_m$. This kinetic behavior was proposed to be due to the exposure of mitochondria to microdomains of high $[Ca^{2+}]_c$ in the regions surrounding the $InsP_3$-sensitive calcium release sites on the ER (3, 4). This model was recently supported by data obtained in freshly isolated hepatocytes using dihydro-Rhod 2 to monitor $[Ca^{2+}]_m$ (16). We provide further evidence to support this hypothesis but demonstrate that this proposal does not hold true for ECV304 cells.

In particular, we show that the $[Ca^{2+}]_m$ increases seen in response to ATP in ECV304 cells are slow-onset sustained signals that are quite insensitive to Ca^{2+} mobilization by $InsP_3$. This differing pattern of $[Ca^{2+}]_m$ may be due to the localization of the mitochondria, which is quite different from other cells, *e.g.* HeLa cells. The ECV304 cell mitochondria are not closely associated with any specific subcellular structure or organelle, whereas in the case of MH75 cells over 50% of the mitochondria lie within 700 nm of the ER. Thus in the ECV304 cells, the increases in $[Ca^{2+}]_m$ seem to depend on global $[Ca^{2+}]_c$ changes and thus appear to be mainly affected by Ca^{2+} influx across the plasma membrane.

MATERIALS AND METHODS

Reagents and Solutions

During all experiments cells were constantly bathed with physiological saline, at 37 °C and pH 7.4, of the following composition: 145 mM

* This work was supported by grants from the Medical Research Council, The Royal Society, The Wellcome Trust, and the University of Liverpool Research Development Fund (to A. W. M. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed. Tel.: (+44) 151 794 5510; Fax: (+44) 151 794 5517; E-mail: awms@liv.ac.uk.

[1] The abbreviations used are: $[Ca^{2+}]_c$, cytosolic free calcium concentration; $[Ca^{2+}]_m$, mitochondrial matrix free calcium concentration; Ca^{2+}_o, extracellular calcium; ECV304, human umbilical vein-derived endothelial cell line; MH75, HeLa cell clone stably transfected with mitochondrially targeted aequorin; $InsP_3$, inositol 1,4,5-trisphosphate; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; ER, endoplasmic reticulum; cytAEQ, cytoplasmic aequorin; mtAEQ, mitochondrial aequorin.

NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 1 mM $MgSO_4$, 10 mM Hepes, 10 mM D-glucose, plus either 1 mM $CaCl_2$ or 0.5 mM EGTA (Ca^{2+}-free). All reagents were added to this buffer as indicated in the text and by the bars on the figures. All chemicals and reagents were purchased from Merck Ltd. (Lutterworth, Leics., U.K.) or Sigma (Poole, Dorset, U.K.) unless otherwise stated.

Plasmids

Both cytoplasmic (untargeted, cytAEQ) and mitochondrially targeted aequorin (mtAEQ) containing plasmids were obtained by the method described previously (2, 12). Extraction and purification from bacterial suspensions were achieved using a Wizard Maxiprep plasmid preparation kit (Promega Ltd., U.K.).

Cell Culture

The human umbilical vein endothelial cell derived line, ECV304, was maintained at 37 °C in a 5% CO_2 humid incubator, in 80-cm^2 flasks containing Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotic, antimycotic solution (Sigma, Poole). A clone of the HeLa cell line, stably transfected with mtAEQ and designated MH75 (3, 4), was maintained in the same way. All tissue culture reagents were purchased from Life Technologies (Renfrewshire, U.K.).

Transient Transfection of Endothelial Cells

One to two days prior to experimentation, ECV304 cells were seeded onto 16-mm glass coverslips (Merck Ltd., Lutterworth), using trypsin/EDTA in 12-well culture plates (Costar). On reaching 50–70% confluence, transient transfection was carried out by calcium phosphate precipitation. Briefly, 4–6 μg of plasmid per coverslip was further purified in ethanol with 23% TE (10 mM Tris-HCl + 1 mM EDTA) buffer, and 2% sodium acetate (3 M, pH 8.0), in an ethanol dry ice bath held at −80 °C. After 30–40 min this mixture was spun at 14,000 rpm in an Eppendorf microcentrifuge for 15 min. The supernatant was carefully removed in a sterile laminar flow cabinet, and the plasmid pellet was resuspended in TE buffer and 10% $CaCl_2$. This was then transferred dropwise, while vortexing, to a tube contained double-concentrated Hepes-buffered saline (2 × HBS, 280 mM NaCl, 50 mM Hepes, 1.5 mM Na_2HPO_4) at precisely pH 7.12. After exactly 30 min the precipitate was visible, and 100 μl of this was then transferred dropwise to each coverslip. Dulbecco's modified Eagle's medium was renewed the following morning, and cells were at maximal expression state the subsequent day (36–48 h after transfection).

Measurement of Mitochondrial Calcium ($[Ca^{2+}]_m$)

Cells transfected with mtAEQ and grown on 16-mm glass coverslips were incubated with 5 μM coelenterazine (Molecular Probes, Eugene, OR) at 37 °C for 1 h and then in a high oxygen atmosphere at 37 °C for a further 1–2 h to favor the regeneration reaction (17). Following this incubation period, coverslips were transferred to a superfusion chamber held in close proximity to a photomultiplier tube, as described previously (18). The output from the photomultiplier tube was then fed directly into a microcomputer and analyzed using OSCAR software (Photon Technologies Inc. (PTI)). Calibration was then performed using AEQASC software kindly provided by Javier Alvarez (19). The perfusion chamber was warmed to 37 °C using a circulating water bath, and solutions were superfused over the coverslip at a rate of 1 ml min^{-1}.

Measurement of Cytosolic Calcium ($[Ca^{2+}]_c$)

Cells transfected with cytAEQ were reconstituted with coelenterazine and monitored in the same way as described above.

Alternatively, $[Ca^{2+}]_c$ was also monitored by loading cells, grown on 22-mm diameter coverslips, with Fura-2AM (Molecular Probes). This was achieved by replacing the growth medium with physiological saline containing 0.1% bovine serum albumin, 2 μM Fura-2AM, and 0.0125% Pluronic F127 detergent (Molecular Probes) and incubating coverslips at 37 °C for 45 min. Coverslips were placed in a purpose-built perfusion chamber where they were constantly superfused, again at a rate of 1 ml min^{-1}. Changes in fluorescence were then monitored using an intensified charge-coupled device camera (Photonic Science Ltd.). The data were analyzed using a PTI digital imaging system equipped with Image Master for Windows software (PTI). This method has the advantage that single cells within a population can be monitored, whereas data from whole coverslip populations are obtained when using cytAEQ transfection.

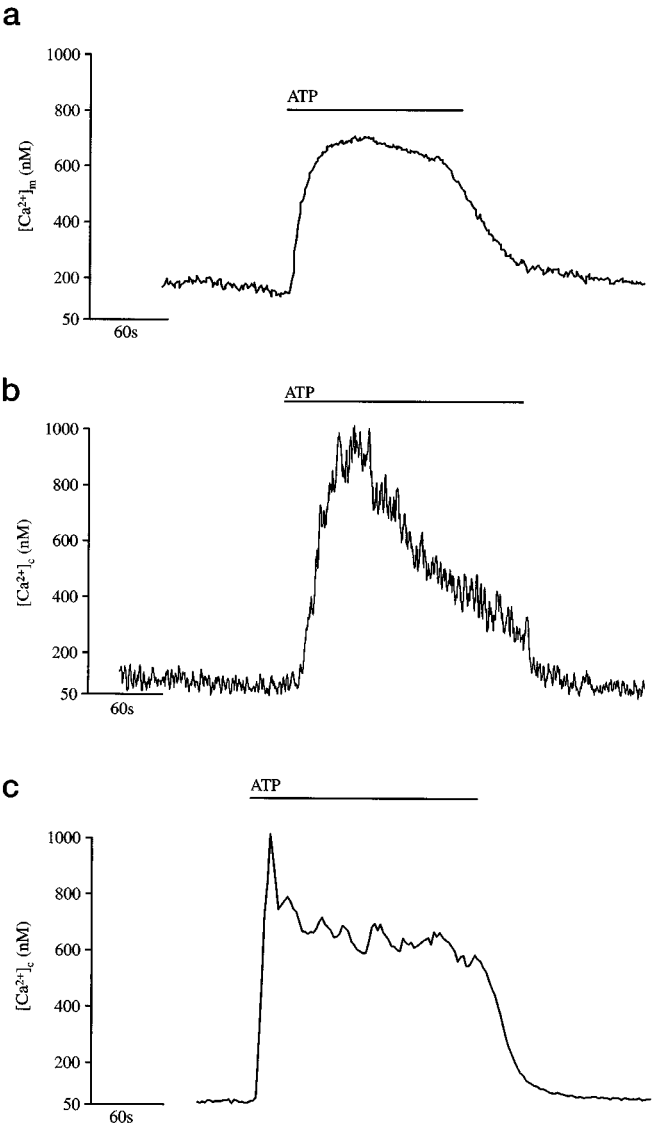

FIG. 1. *a*, mitochondrial calcium changes ($[Ca^{2+}]_m$) were measured in mtAEQ transiently transfected ECV304 endothelial cells. Applications of 10 μM ATP for long periods resulted in sustained $[Ca^{2+}]_m$ increases that are comparable with changes in $[Ca^{2+}]_c$ as shown in *b* and *c*. $[Ca^{2+}]_c$ changes in ECV304 cells in response to ATP were measured using cytAEQ (*b*) (10 μM ATP) or Fura-2 (*c*) (1 μM ATP). For *a* and *b*, points were obtained every 200 ms; for *c*, images were captured every second resulting in ratio images every 2 s. *a* and *b*, the trace represents $[Ca^{2+}]$ measured from the whole coverslip; *c*, the data are the mean of 20 single Fura-2-loaded cells. *Bars* represent the presence of ATP in the perfusion medium; 1 mM Ca^{2+} was present throughout.

Electron Micrographs

Cells were prepared for fixation in one of two formats as follows.

Pellet Fixation—Cells from an 80-cm^2 flask were dislodged using trypsin/EDTA and placed in a sterile microcentrifuge tube. The suspension was spun at 1000 rpm (80 × *g*) for 2 min, after which they were fixed and stained as indicated below.

Cultured Cells—Cells were grown on small squares (2 × 2 cm) of Mellinex 228 sheeting, this is softer than glass coverslips and facilitates sectioning. Once 90–100% confluent the sheets were fixed and stained as indicated below.

Fixing and Staining—Cells were primarily fixed in 2.5% glutaraldehyde and 2.0% formaldehyde in 0.1 N sodium cacodylate (NaCac) buffer at pH 7.3 and 3 °C, after which they were washed twice in NaCac buffer at pH 7.3 and 3 °C. A postfixation was then performed in 2.0% osmium tetroxide in NaCac buffer at pH 7.3 and 3 °C and then washed twice in 0.15 N sodium acetate (NaAc) buffer at pH 7.3 and 3 °C. The preparation was then stained in 1.0% uranyl acetate in NaAc buffer at pH 7.3, 25 °C to highlight membranes. Dehydration in increasing concentrations of

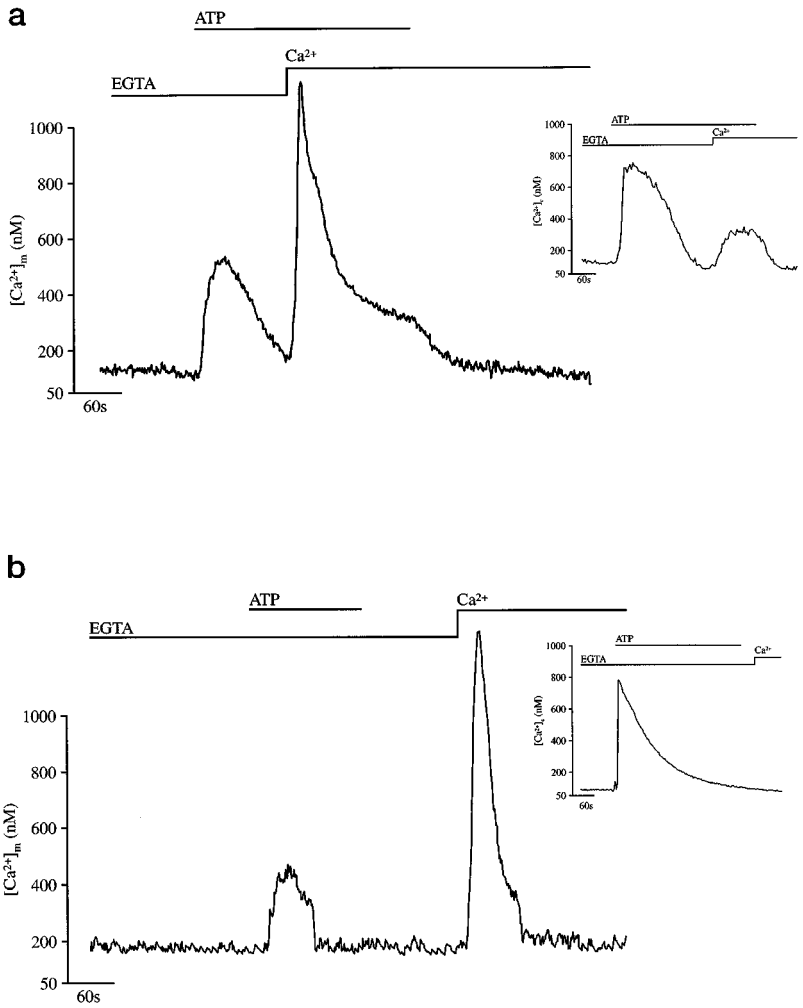

FIG. 2. **Comparison of the effect of readdition of Ca^{2+}_o either during (*a*) or following (*b*) stimulation with 10 μM ATP for $[Ca^{2+}]_m$ and $[Ca^{2+}]_c$ (*insets*) in ECV304 cells.** Data points were obtained every 200 ms for $[Ca^{2+}]_m$ traces and every 2 s for $[Ca^{2+}]_c$ traces. *Bars* represent the addition of reagents to the perfusion bath. 0.5 mM EGTA was present until 1 mM Ca^{2+} was added where indicated.

ethanol (10, 30, 50, 70, 90, and 100%) was then performed, including a final second step in 100% ethanol. Samples were infiltrated with increasing concentrations of LR White™ resin (London Resin Co., U.K.) (50, 75 and 100%) finishing with three applications of 100% LR White™ for a total of 4 h. Samples were finally embedded in LR White™ resin for 48 h at 60 °C. Secondary staining was performed on the grid to highlight membranes further when necessary. This was performed using 2% uranyl acetate in 30% methanol for 1 min, followed by a wash in distilled water, then a 1-min incubation in 10% lead citrate, and a final wash and dry.

RESULTS

Changes in Mitochondrial Calcium ($[Ca^{2+}]_m$) in Response to ATP—When the transfected human umbilical vein endothelial cell-derived line ECV304 was stimulated with ATP (1–10 μM), a slow and sustained rise in $[Ca^{2+}]_m$ was observed (Fig. 1*a*). The time to half-maximal ($t_{½}$) for the rise was 18.6 ± 1.3 s (x ± S.E.) (n = 11), and $[Ca^{2+}]_m$ rose to between 700 nM and 1 μM, rarely exceeding 1 μM. $[Ca^{2+}]_m$ remained elevated for the duration of agonist presence, *i.e.* for up to 10 min in some cases (Fig. 1*a*). The increase in $[Ca^{2+}]_m$ was comparable with the $[Ca^{2+}]_c$ response in these cells (Fig. 1, *b* and *c*) and contrasts dramatically with the rapid and large increase in $[Ca^{2+}]_m$ seen in hepatocytes and several other cell lines (12, 16,).

When cells were treated with the mitochondrial uncoupling agent FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, 1 μM), the response was significantly attenuated. $\Delta[Ca^{2+}]_m$ for ATP alone was 1070 ± 136 nM as compared with a $\Delta[Ca^{2+}]_m$ for ATP and FCCP of only 185 ± 61 nM ($p < 0.001$, n = 6). In two of the six experiments, there was no detectable ATP-evoked rise in $[Ca^{2+}]_m$ in the presence of FCCP. The effects seen with FCCP were reversible following a short period of wash and readdition of ATP (n = 6). While FCCP had this dramatic effect on $[Ca^{2+}]_m$, it was found to have no effect of the ATP-evoked rise in $[Ca^{2+}]_c$ (n = 21). Examination of transfected cells by immunofluorescent localization of the mitochondrially targeted aequorin revealed a similar reticular pattern of fluorescence to that previously seen for MH75 cells (19). These observations confirm that the detected rise in response to ATP is a true reflection of the rise in $[Ca^{2+}]_m$.

Following an initial stimulation of ECV304 cells with ATP, a brief wash followed by subsequent applications of agonist resulted in comparable rises in $[Ca^{2+}]_m$ with only a modest reduction in magnitude (n = 11, not shown).

Changes in Cytosolic Calcium ($[Ca^{2+}]_c$) in Response to ATP—The unusual kinetics of $[Ca^{2+}]_m$ in ECV304 cells in response to ATP prompted us to investigate whether this behavior was due to some peculiarities of the $[Ca^{2+}]_c$ response in this cell type. This was monitored using either untargeted aequorin (Fig. 1*b*, n = 9) or Fura-2 (Fig. 1*c*, n = 20). In fact, stimulation of ECV304 cells, with ATP (1–10 μM), evoked a rise in $[Ca^{2+}]_c$ (n = 40), which was quite typical both in terms of kinetics, with a $t_{½}$ of 2.12 ± 0.62 s for 1 μM (n = 20) and 1.97 ± 0.58 s for 10 μM (n = 20) ($t_{½}$ for 1 μM is not significantly different to that for 10 μM ($p > 0.05$)), and of amplitude (mean $[Ca^{2+}]_c$ for 1 μM ATP = 906.3 ± 27.2 nM (n = 20) and for 10 μM = 933.6 ± 53.2 nM (n = 20) which are not significantly different from one another ($p > 0.05$)). The typical biphasic nature is revealed by stimulation in the absence of extracellular calcium (following a 2–4-min prewash in 0.5 mM EGTA), which led to a transient response (n =

11) (Fig. 2*b*, *inset*) with an equivalent $t_{½}$ (1.61 ± 0.11 s) and maximal $[Ca^{2+}]_c$ (955.9 ± 36.7 nM). In the presence of $Ca^{2+}{}_o$ the response is sustained for as long as the agonist is present (Fig. 1, *b* and *c*). Repetitive applications of ATP each evoke a comparable response with only a small degree of reduction, presumably due to receptor down-regulation, provided calcium is present in the extracellular medium (not shown).

The Effects of Calcium Influx on $[Ca^{2+}]_m$—Because of the sustained nature of the rise in $[Ca^{2+}]_m$ in response to ATP, the dependence on $Ca^{2+}{}_o$ was investigated. Washing the cells in buffer supplemented with 0.5 mM EGTA (calcium-free) and subsequent stimulation with ATP result in a significantly lower rise in $[Ca^{2+}]_m$. $\Delta[Ca^{2+}]_m$ for ATP + EGTA = 280 ± 43.3 nM (Fig. 2, *a* and *b*), whereas control ATP responses are nearly three times as large with a $\Delta[Ca^{2+}]_m$ of 763 ± 63.6 nM ($p < 0.001$, $n = 14$). Two of the fourteen experiments in 0.5 mM EGTA resulted in no rise above basal $[Ca^{2+}]_m$. These small responses were extremely transient with a duration of only 62.2 ± 6 s ($n = 12$).

Replacement of $Ca^{2+}{}_o$ during ATP stimulation, while monitoring $[Ca^{2+}]_m$, in ECV304 cells ($n = 11$), led to a second rapid rise in $[Ca^{2+}]_m$ (Fig. 2*a*), which was significantly higher magnitude than the increase elicited by agonist addition in Ca^{2+}-free medium (315.8 ± 89% of initial increase $n = 8$, $p < 0.001$). In contrast Fig. 2*a*, *inset*, shows results of the same protocol while monitoring $[Ca^{2+}]_c$. In 8 of the 11 experiments, the restoration of the response was of a magnitude comparable with the plateau phase of a normal biphasic sustained rise in $[Ca^{2+}]_c$ (Fig. 2*a*, *inset*). In less than 30% (3 experiments) of cells the rise in $[Ca^{2+}]_c$ obtained upon Ca^{2+} readdition was equal to, or very slightly higher than, the initial rise in response to ATP in EGTA-containing medium.

An even more dramatic dissociation between $[Ca^{2+}]_m$ and $[Ca^{2+}]_c$ was observed using the protocol employed in Fig. 2*b*. In this latter case, the cells were first stimulated with ATP, in Ca^{2+}-free, EGTA-containing medium. The agonist was then washed away and the $Ca^{2+}{}_o$ added back a few minutes later. Ca^{2+} readdition led to a large, transient rise in $[Ca^{2+}]_m$ (Fig. 2*b*; $n = 6$), whereas a rise was not evident upon $Ca^{2+}{}_o$ replacement when monitoring $[Ca^{2+}]_c$ ($n = 5$, Fig. 2*b*, *inset*) or $[Ca^{2+}]_m$ in the presence of 1 μM FCCP ($n = 5$, not shown). Following the restoration of $Ca^{2+}{}_o$, and a brief wash period, subsequent addition of ATP produced the typical sustained rise in $[Ca^{2+}]_m$, indicating that during the experiment there had been no marked desensitization of the ATP response ($n = 14$). In addition, application of the receptor-mediated Ca^{2+} entry blocker SKF96365 (50 μM) (20) completely abolished the rise in $[Ca^{2+}]_m$ in all cases ($n = 6$).

$[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ Responses in MH75 Cells—Under identical experimental conditions, MH75 cells stimulated with ATP show an increase in $[Ca^{2+}]_m$ in the order of 2–3 μM as opposed to ≤1 μM in ECV304 cells. However, the rise in $[Ca^{2+}]_c$ seen in these cells was comparable with that seen in ECV304 cells (*i.e.* submicromolar). The $t_{½}$ for $[Ca^{2+}]_m$ in MH75 cells in response to ATP was 4.0 ± 0.5 s, and the duration was only 71.7 ± 10 s even though the agonist remained present in the perfusion medium for several minutes. Such a short rise time is significantly different from that seen in response to ATP in ECV304 cells ($p < 0.005$). These responses are similar to those previously reported for histamine-stimulated MH75 cells (3, 4), and there is no significant difference between either duration ($p = 0.5$) or $t_{½}$ ($p = 0.5$) for ATP or histamine-evoked changes in $[Ca^{2+}]_m$ in MH75 cells. Stimulation of these cells in the absence of $Ca^{2+}{}_o$ evokes a similar response but, in contrast to the ECV304 cells, replacing $Ca^{2+}{}_o$ results in a secondary rise very much smaller than in the first (Fig. 3*a*, $n = 6$). The same

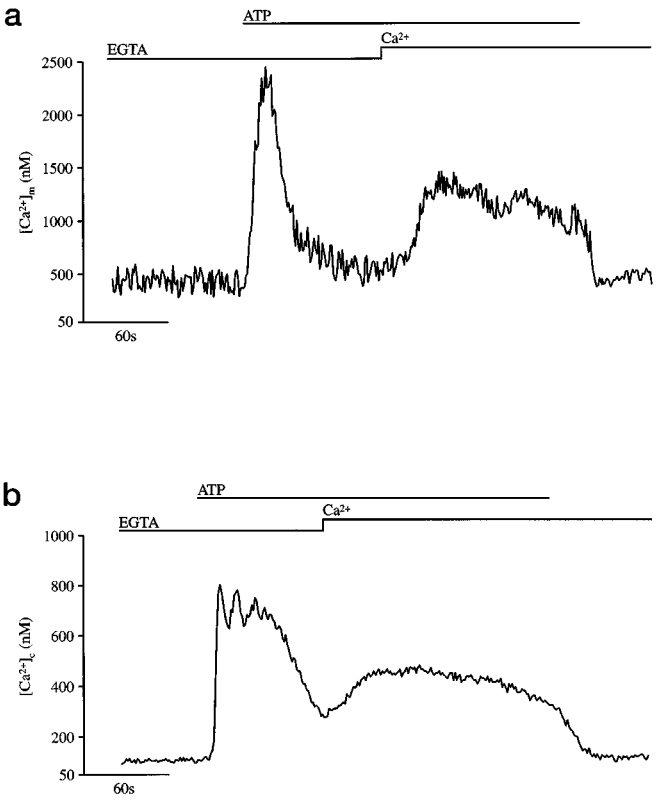

FIG. 3. **Comparison of the readdition of $Ca^{2+}{}_o$ during stimulation with 10 μM ATP between $[Ca^{2+}]_m$ (*a*) and $[Ca^{2+}]_c$ (using Fura-2) (*b*) for MH75 cells.** 0.5 mM EGTA was present to start until 1 mM Ca^{2+} was applied as indicated by the *bars*. The presence of ATP is also indicated by the *bars*. Points were obtained every 200 ms for *a*, and images were captured every 2 s for *b*.

protocol was applied while monitoring $[Ca^{2+}]_c$ (Fig. 3*b*, $n = 12$) and revealed comparable results to the ECV304 cell $[Ca^{2+}]_c$ response in Fig. 2*b*.

Localization of Mitochondria—Electron micrographs were prepared of both MH75 and ECV304 cells from pellets of cells harvested from tissue culture flasks (Fig. 4 *a* and *c*), and also from monolayers grown on Mellinex 228 sheeting (Fig. 4, *b* and *d*). Since there was no difference in the observed distribution of structures using either preparation method, the data from both sets of micrographs were pooled.

The micrographs in Fig. 4, *a* and *b*, show the distribution of mitochondria in ECV304 cells, at 90,000 and 60,000 ×, respectively. It is evident that while mitochondria are abundant, there is little ER in these cells. Fig. 4, *c* and *d*, shows the distribution of mitochondria in MH75 cells, at 90,000 and 40,000 ×, respectively. Abundant ER is visible, which appears to be in close association with the mitochondria even when near to the plasma membrane. Compare Fig. 4*b* for ECV304 with Fig. 4*d* for MH75 cells.

To establish the localization of subcellular structures in respect to mitochondria, the number of "hits" with each structure of interest (plasma membrane, ER, or nuclear envelope) met along rings at set distances (100 nm to 1 μm, step 100 nm) from the center of a given mitochondrion was counted. The number of mitochondria included for MH75 cells was 84 and 107 for ECV304 cells. The resulting probability of meeting either endoplasmic reticulum (Fig. 5*a*) or plasma membrane (Fig. 5*b*) at each distance has been plotted for both cell types in Fig. 5. The size of mitochondria varied but was never less than 300 nm (*i.e.* <300 nm from center was still mitochondria). In ECV304 cells, there was a much lower probability of meeting ER within a short distance (<1 μm) from the mitochondria than in the

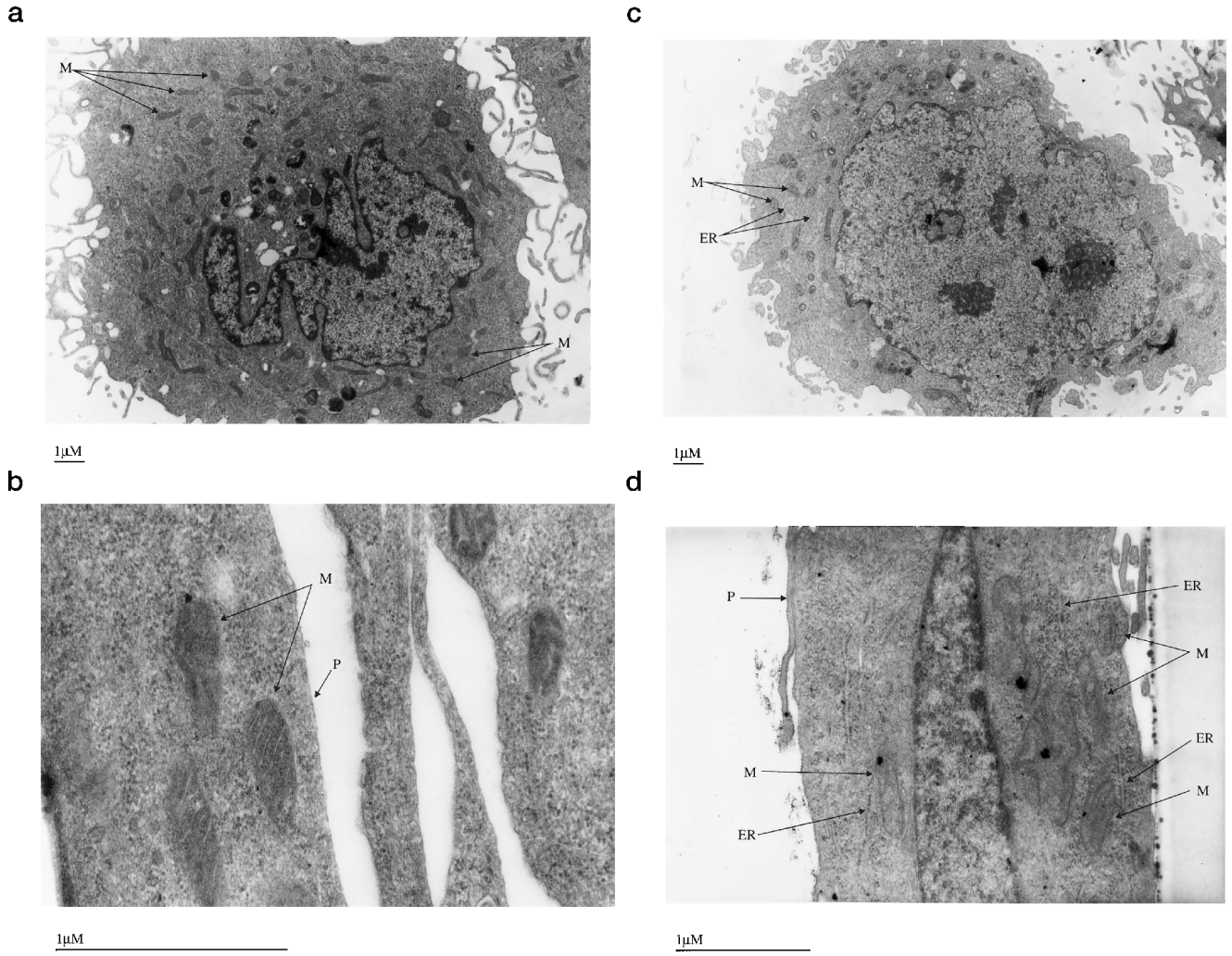

FIG. 4. **Electron micrographs of MH75 and ECV304 demonstrating the localization of mitochondria and membranous organelles.** *a* and *b*, ECV304 cells at 90,000 and 60,000 × magnification, respectively. *c* and *d*, MH75 cells at 90,000 and 40,000 × magnification, respectively. *a* and *c* are from pelleted cells, and *b* and *d* are taken from cells grown on Mellinex 228 sheeting. *M*, mitochondria; *P*, plasma membrane; *ER*, endoplasmic reticulum.

MH75 cells (Fig. 5*a*). Fig. 5*b* shows that mitochondria in the ECV304 cells were more likely to be close to the plasma membrane than in the MH75 cells, but these probabilities are still less than those for the MH75 mitochondrial relationship with ER (*e.g.* the probability of MH75 mitochondria being within 700 nm of ER is >0.5, whereas the probability of ECV304 cell mitochondria being within the same distance of the plasma membrane is <0.15).

Counting the total number of mitochondria situated with their centers close to ER or plasma membrane reveals that only 3.8% of the ECV304 cell mitochondria were within 700 nm of the ER, compared with 75% of MH75 cell mitochondria. In contrast, 14% of the ECV304 cell mitochondria lie within the same distance of the plasma membrane whereas only 5.6% were similarly located in MH75 cells.

In Fig. 5, *c* and *d*, the probability of meeting ER was compared with the probability of meeting plasma membrane for ECV304 cells (Fig. 5*c*) and MH75 cells (Fig. 5*d*). It can be seen from this that within 900 nm of the mitochondrial center, in ECV304 cells, there is a much higher chance of meeting the plasma membrane than any ER membrane (Fig. 5*c*), which is in contrast to the case in the MH75 cells (Fig. 5*d*). At 900 nm to 1 μm the likelihood of meeting either organelle becomes equal (Fig. 5*c*).

The probability of meeting the nuclear envelope was also measured and was found to be the same for both cell types, *i.e.* very low at short distances (<600 nm) and increasing to only 0.15 at 1 μm (not shown).

From these data, it would appear that although the mitochondria are distributed throughout the cytosol in both cell types, the probability of their being close to the ER is considerably lower in the ECV304 cells than in the MH75 cells. In addition, in the ECV304 cells there is a higher chance of the mitochondria being situated close to the plasma membrane than to the ER.

DISCUSSION

The large, rapid increase in $[Ca^{2+}]_m$ evoked by $InsP_3$-generating agonists was one of the most surprising new pieces of information provided by the technique of recombinant, organelle-targeted, aequorins (2–4, 12). For over a decade the general consensus was that, under physiological conditions, mitochondria should play a minor role in the control of Ca^{2+} homeostasis (7), given the low affinity of the uniporter and the relatively small increases in $[Ca^{2+}]_c$ elicited by receptor stimulation. This apparent paradox has been explained by the hypothesis that microdomains of $[Ca^{2+}]_c$ are sensed by mitochondria localized in the vicinity of the Ca^{2+} release sites (3, 4). This hypothesis has been further supported by recent data obtained in freshly isolated hepatocytes using dihydro-Rhod 2

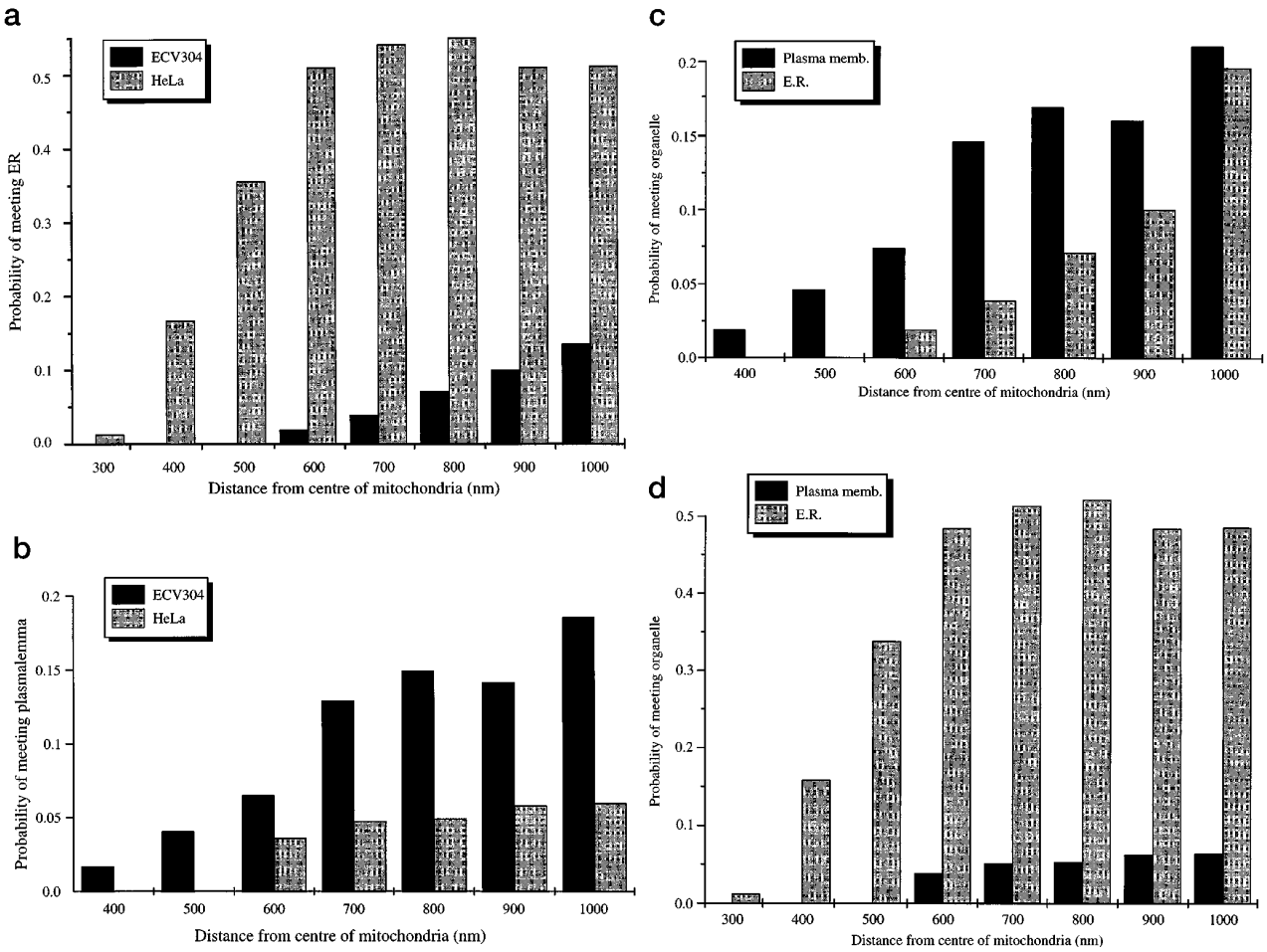

FIG. 5. *a* shows the probability of meeting ER at each fixed distance from the center of a mitochondrion and compares ECV304 cells (*dark bars*) with MH75 cells (*light bars*). *b* compares the probability of meeting plasma membrane at each distance between ECV304 (*dark bars*) and MH75 cells (*light bars*). *c* compares the probability of meeting ER (*light bars*) with the probability of meeting plasma membrane (*dark bars*) in ECV304 cells. *d* shows a similar comparison for MH75 cells. All probability values were calculated by dividing the number of hits for each subcellular structure with the total number of structures encountered at each fixed distance from the center of the mitochondrion.

or the intrinsic fluorescence of NAD(P)H (16). Thus, in all cell types analyzed so far, the kinetics of $[Ca^{2+}]_m$ changes in response to agonist stimulation appear to be rapid and large increases that decline to prestimulatory levels faster than $[Ca^{2+}]_c$.

The data presented here for the endothelial cell line ECV304 are, at a first sight, in contradiction with the microdomain hypothesis. In this cell type addition of extracellular ATP in the presence of $Ca^{2+}{}_o$ evokes a slow-onset but sustained rise in $[Ca^{2+}]_m$ that can be reversibly inhibited by treatment with the mitochondrial uncoupling agent FCCP. Furthermore, in contrast to MH75 and other cell types, stimulation in the absence of $Ca^{2+}{}_o$ results in a drastic reduction, and in some cases even abolition, of the $[Ca^{2+}]_m$ increases, suggesting that the measured rise in $[Ca^{2+}]_m$ may be critically dependent upon Ca^{2+} influx rather than the release of Ca^{2+} from intracellular stores.

Because $[Ca^{2+}]_c$ responses are comparable between ECV304 cells and MH75 cells, why does agonist stimulation not evoke the large, rapid rise in $[Ca^{2+}]_m$ characterized in other cell types? This question may, in part, be answered by the electron microscopic data. Here we show that a very high degree of association exists between the mitochondria and the ER in the MH75 cells. However, there is no such close association between these two organelles in the ECV304 cells; indeed, $<4\%$ of the mitochondria in these cells are within 700 nm of the ER. In ECV304 cells, therefore, the mitochondria are not, as in the HeLa cells, located in sufficiently close proximity to the ER to detect any $[Ca^{2+}]_c$ microdomains which would occur at the time of agonist-stimulated Ca^{2+} release.

The ATP-stimulated slow-onset sustained rise in $[Ca^{2+}]_m$ is 2–3-fold smaller in magnitude than the $[Ca^{2+}]_m$ responses seen in other nonexcitable cells (≤ 1 μM as opposed to 2–3 μM in the MH75 cells) (3, 4). A similar type of small, slow-onset response can be obtained in MH75 cells and hepatocytes when the ER Ca^{2+}-ATPase inhibitors *t*-butyl-benzohydroquinone (21) or thapsigargin are used to promote a sustained rise in $[Ca^{2+}]_c$ (4, 16, 19) via an $InsP_3$-independent process involving Ca^{2+} influx rather than the rapid release from intracellular stores. Such responses lend support to the suggestion that the slow, sustained $[Ca^{2+}]_m$ response in ECV304 cells is maintained by Ca^{2+} influx.

It remains to be established why in ECV304 cells a sustained increase in bulk $[Ca^{2+}]_c$ elicited by receptor stimulation is more effective at maintaining a sustained increase in $[Ca^{2+}]_m$ than in HeLa or hepatocytes. Several not mutually exclusive alternatives can be suggested. (i) The large, rapid rise in $[Ca^{2+}]_m$ triggered by receptor stimulation in HeLa and other cells (3, 4, 16) could, itself, be responsible for the transient nature of their responses. The high $[Ca^{2+}]_m$ may induce run-down of the mitochondrial Ca^{2+} uniporter by a process analogous to the Ca^{2+}-dependent run-down seen in voltage-gated Ca^{2+} channels (22–24). (ii) The H^+:Ca^{2+} and Na^+:Ca^{2+} antiporters (in highly responding cells) could undergo a prolonged activation by the large increase in $[Ca^{2+}]_m$, in a manner similar to the Ca^{2+}

activation of the plasma membrane Na^+:Ca^{2+} exchanger (25, 26). The absence of a large initial $[Ca^{2+}]_m$ spike in the ECV304 cell mitochondria, and in the *t*-butyl-benzohydroquinone- or thapsigargin-stimulated cells, would therefore favor the sustained elevation in $[Ca^{2+}]_m$ that is observed.

The electron microscopy studies point out another difference between ECV304 cells and the HeLa MH75 clone (previously characterized in detail for their $[Ca^{2+}]_m$ responses) as far as mitochondria localization is concerned. A small proportion of the mitochondria in ECV304 cells is localized within a relatively small distance (<800 nm) of the plasma membrane. Although the response to the release of stored Ca^{2+} is quite small, readdition of Ca^{2+} in ECV304 cells, either in the presence or absence of ATP, causes a rapid and transient elevation of $[Ca^{2+}]_m$ that is not reflected in the $[Ca^{2+}]_c$. It is tempting to speculate that this association between mitochondria and plasma membrane is at the basis of the fast and relatively large response of these cells under conditions where the influx of Ca^{2+} from the extracellular medium is maximized, *i.e.* by first depleting the cells in EGTA medium followed by Ca^{2+} readdition. A similar effect is not observed in $[Ca^{2+}]_m$ or $[Ca^{2+}]_c$ in MH75 cells. This suggests that an influx of $Ca^{2+}{}_o$ may generate a microdomain of high $[Ca^{2+}]_c$ close to the plasma membrane causing a rapid uptake of Ca^{2+} into the mitochondria located in this region.

Taken together the results described in this study lead to a few general considerations as shown. (i) The kinetics and amplitude of the $[Ca^{2+}]_m$ increases depend on the cell type and are due to the structural relationships between mitochondria and the source of $[Ca^{2+}]_c$ increases. (ii) Fast increases in $[Ca^{2+}]_m$ are always dependent on $[Ca^{2+}]_c$ microdomains; whether these rapid increases are triggered by Ca^{2+} release from stores or by Ca^{2+} influx depends on the preferential vicinity of the mitochondria to the ER or to the plasma membrane, respectively. Thus, in any cell type, different modes of communication between cytosolic and mitochondrial Ca^{2+} can exist. For example, in cell types where there is a close association between mitochondria and ER, a sustained elevation of $[Ca^{2+}]_m$, and thus a prolonged activation of the dehydrogenases, can be elicited by high frequency $[Ca^{2+}]_c$ oscillations, as shown by Hajnóczky *et al.* (16) in hepatocytes; alternatively, as in the case of ECV304 cells, the same result can be obtained by a prolonged elevation of $[Ca^{2+}]_c$. (iii) Last, but not least, the present data predict that in cell types where a major source of $[Ca^{2+}]_c$ elevation is via a large Ca^{2+} influx current through plasma membrane Ca^{2+} channels, there may be a selective activation of subpopulations of mitochondria in the proximity of the channels themselves. Preliminary data from our laboratory[2] indicate that this is indeed the case. The possibility of different ways of controlling mitochondrial Ca^{2+} homeostasis offers a unique flexibility to those signaling mechanisms that may have important physiological consequences. For example, as far as endothelial cells are concerned, since the regulation of blood vessel tone is a long term physiological effect, the cells will be required to release vasodilators such as nitric oxide for a prolonged period. This, along with the maintenance of the $[Ca^{2+}]_c$ signal, will require a sustained supply of energy in the form of ATP and a large production of L-arginine for NO synthesis. Depending on the agonist and the individual cell type this might be achieved by either high frequency oscillations or long lasting increases of $[Ca^{2+}]_m$, which we observed here.

[2] R. Rizzuto and T. Pozzan, unpublished data.

Acknowledgments—We would like to thank Marisa Brini for her assistance in the laboratory in Italy, Brian Boothroyd for his invaluable expertise taking the electron micrographs, and Dave Bassi for photography. We would also like to thank Tracie Walker and Alan Conant for their help in preparing the manuscript.

REFERENCES

1. Berridge, M. J. (1993) *Nature* **361,** 315–325
2. Rizzuto, R., Simpson, A. W. M., Brini, M., and Pozzan, T. (1992) *Nature* **358,** 325–327
3. Rizzuto, R., Brini, M., Murgia, M., and Pozzan, T. (1993) *Science* **262,** 744–747
4. Rizzuto, R., Bastianutto, C., Brini, M., Murgia, M., and Pozzan, T. (1994) *J. Cell Biol.* **126,** 1183–1194
5. Denton, R. M., and McCormack, J. G. (1990) *Annu. Rev. Physiol.* **52,** 451–466
6. McCormack, J. G., and Denton, R. M. (1993) *Biochem. Soc. Trans.* **21,** 793–799
7. Carafoli, E. (1987) *Annu. Rev. Biochem.* **56,** 395–433
8. Gunter, T. E., and Pfeiffer, D. R. (1990) *Am. J. Physiol.* **258,** C755–C786
9. McCormack, J. G., Daniel, R. L., Osbaldeston, N. J., Rutter, G. A., and Denton, R. M. (1992) *Biochem. Soc. Trans.* **20,** 153–159
10. Rizzuto, R., Bernadi, P., Favaron, M., and Azzone, G. F. (1987) *Biochem. J.* **246,** 271–277
11. Miyata, H., Silverman, H. S., Sollott, S. J., Lakatta, E. G., Stern, M. D., and Hansford, R. G. (1991) *Am. J. Physiol.* **261,** H1123–H1134
12. Rizzuto, R., Brini, M., and Pozzan, T. (1994) *Methods Cell Biol.* **40,** 339–358
13. Takahashi, K., Sawasaki, Y., Hata, J.-I., Mukia, K., and Goto, T. (1990) *In Vitro Cell Dev. Biol.* **25,** 265–274
14. Moncada, S., Palmer, R. M. J., and Higgs, E. A. (1991) *Pharmacol. Rev.* **43,** 109–142
15. Newby, A. C., and Henderson, A. H. (1990) *Annu. Rev. Physiol.* **52,** 661–674
16. Hajnóczky, G., Robb-Gaspers, L. D., Seitz, M. B., and Thomas, A. P. (1995) *Cell* **82,** 415–424
17. Shimomura, O., Musicki, B., and Kishi, Y. (1988) *Biochem. J.* **251,** 405–410
18. Cobbold, P. H., and Lee, J. A. C. (1990) in *Cellular Calcium: A Practical Approach* (McCormack, J. G., and Cobbold, P. H., eds) pp. 55–82, IRL Press, Oxford
19. Brini, M., Marsault, R., Bastianutto, C., Alvarez, J., Pozzan, T., and Rizzuto, R. (1995) *J. Biol. Chem.* **270,** 9896–9903
20. Merritt, J. E., Armstrong, W. P., Benham, C. D., Hallam, T. J., Jacob, R., Jaxa-Chamiec, A., Leigh, B. K., McCarthy, S. A., Moores, K. E., and Rink, T. J. (1990) *Biochem. J.* **271,** 515–522
21. Kass, G. E. N., Duddy, S. K., Moore, G. A., and Orrenius, S. (1989) *J. Biol. Chem.* **264,** 15192–15198
22. Giannattasio, B., Jones, S. W., and Scarpa, A. (1991) *J. Gen. Physiol.* **98,** 987-1003
23. Köhr, G., and Mody I. (1991) *J. Gen. Physiol.* **98,** 941–967
24. Yue, D. T., Backx, P. H., and Imredy, J. P. (1990) *Science* **250,** 1735–1738
25. Blaustein, M. P., and Santiago, E. M. (1977) *Biophys. J.* **20,** 79–111
26. Rasgado-Flores, H., and Blaustein, M. P. (1987) *Am. J. Physiol.* **252,** C499–C504

A Role for Calcium Influx in the Regulation of Mitochondrial Calcium in Endothelial Cells

Alison M. Lawrie, Rosario Rizzuto, Tullio Pozzan and Alec W. M. Simpson

J. Biol. Chem. 1996, 271:10753-10759.
doi: 10.1074/jbc.271.18.10753