Mitochondria respond to Ca\(^{2+}\) already in the submicromolar range: correlation with redox state

J. G. Pitter,1 P. Maechler,2 C. B. Wollheim,2 A. Spät1

1Department of Physiology and Laboratory of Cellular and Molecular Physiology, Semmelweis University, Faculty of Medicine, PO box 259, H-1444, Budapest, Hungary
2Division of Clinical Biochemistry, Department of Internal Medicine, University of Geneva, CH-1211 Geneva, Switzerland

Summary Rapid formation of high-Ca\(^{2+}\) perimitochondrial cytoplasmic microdomains has been shown to evoke mitochondrial Ca\(^{2+}\) signal and activate mitochondrial dehydrogenases, however, the significance of submicromolar cytoplasmic Ca\(^{2+}\) concentrations in the control of mitochondrial metabolism has not been sufficiently elucidated. Here we studied the mitochondrial response to application of Ca\(^{2+}\) at buffered concentrations in permeabilized rat adrenal glomerulosa cells, in an insulin-producing cell line (INS-1/EK-3) and in an osteosarcoma cell line (143Bm\( A\)-13). Mitochondrial Ca\(^{2+}\) concentration was measured with the fluorescent dye rhod-2 and, using an in situ calibration method, with the mitochondrially targeted luminescent protein mt-aequorin. In both endocrine cell types, mitochondrial Ca\(^{2+}\) concentration increased in response to elevated cytoplasmic Ca\(^{2+}\) concentration (between 60 and 740 nM) and an increase in mitochondrial Ca\(^{2+}\) concentration could be revealed already at a cytoplasmic Ca\(^{2+}\) concentration step from 60–140 nM. Similar responses were observed in the osteosarcoma cell line, although a clearcut response was first observed at 280 nM extramitochondrial Ca\(^{2+}\) only. As examined in glomerulosa cells, graded increases in cytoplasmic Ca\(^{2+}\) concentration were associated with graded increases in the reduction of mitochondrial pyridine nucleotides, consistent with Ca\(^{2+}\)-dependent activation of mitochondrial dehydrogenases. Our data indicate that in addition to the recognized role of high-Ca\(^{2+}\) cytoplasmic microdomains, also small Ca\(^{2+}\) signals may influence mitochondrial metabolism. © 2002 Elsevier Science Ltd. All rights reserved.
assumption is in harmony with the low affinity of the mitochondrial Ca\(^{2+}\) uniporter for Ca\(^{2+}\) (K\(_a\) \approx 10^{-5}\) M), the positive cooperative effect of cytoplasmic Ca\(^{2+}\) on the uniporter as well as the rapid saturation of the Ca\(^{2+}\)-extruding mechanisms [22]. Nevertheless, direct [19] and indirect measurements of [Ca\(^{2+}\)]\(_m\) [12,23,24] in adrenomedullary and neural cells indicate that submicromolar Ca\(^{2+}\) signals may also induce mitochondrial Ca\(^{2+}\) signal. Yet recent reviews still ignore or doubt the possibility that submicromolar Ca\(^{2+}\) may influence mitochondrial function.

In rat luteal cells, Ca\(^{2+}\) at low submicromolar concentrations increases [Ca\(^{2+}\)]\(_m\) and the formation of mitochondrial NADPH [25]. Considering that NADPH, a cofactor of steroid hydroxylation, is reoxidized at an increased rate during Ca\(^{2+}\)-induced steroid production [10], it was reasonable to assume that the high Ca\(^{2+}\) sensitivity of mitochondria is a phenomenon specific for steroid-producing cells [26]. The purpose of the present study was to examine whether the mitochondrial responsiveness to Ca\(^{2+}\) in the low submicromolar range is really unique for luteal cells or if it is a more general phenomenon. We examined the mitochondrial response of two endocrine and one non-endocrine cell types: adrenal glomerulosa cells, insulin-producing INS-1/EK-3 cells and the osteosarcoma 143BmA-13 cells. Irrespective of the different role of mitochondrial function in these three cell types, the mitochondrial response to Ca\(^{2+}\) in the low submicromolar range, i.e. just above the resting Ca\(^{2+}\) level, has been clearly observed.

**MATERIALS AND METHODS**

**Materials**

Collagenase was purchased from Millipore (Freehold, NJ, USA), rhod-2 acetoxymethyl ester was from Teflabs (Austin, TX, USA) and coelenterazine was from Molecular Probes (Eugene, OR, USA). Purified Ruthenium Red 360 was provided by Dr P. Enyedi (Budapest, Hungary). Other chemicals were obtained from Sigma (St Louis, MO, USA), Fluka (Buchs, Switzerland), Gibco (Paisley, Scotland, UK) or Calbiochem (Luzern, Switzerland).

**Cell preparation**

Male Wistar rats were used for obtaining glomerulosa cells with the approval (No. 17/1998) of Animal Care and Ethics Committee of the Semmelweis University. The cells were prepared from the adrenal capsular tissue by collagenase digestion, as described previously [27]. The cells were plated on poly-L-lysine-coated glass coverslips in a mixture (38:62, v/v) of modified Kreb's-Ringer-bicarbonate-glucose solution and M199. The mixture contained 3.6 mM K\(^+\), 1.2 mM Ca\(^{2+}\), 0.5 mM Mg\(^{2+}\), 5 mM Hepes and 20 mM HCO\(_3\)\(^-\). The cells were kept for 3–10 h at 37°C under 5% CO\(_2\) (pH 7.4).

The rat insulin-secreting INS-1 cells, stably expressing the Ca\(^{2+}\)-sensitive luminescent photoprotein aequorin targeted to the mitochondria (mt-aequorin), termed INS-1/EK-3, were cultured in RPMI-1640 medium supplemented with 5% fetal calf serum, and other additions, as described previously [11]. The cells were trypsinized 1–4 days before the experiment; 2 \times 10^5 cells were allowed to attach to coverslips and the incubation was continued under identical conditions.

143BmA-13 cells, derived from a human osteosarcoma (ATCC, Number CRL-8303) and stably transfected with mt-aequorin (PM, unpublished results), were cultured similarly to INS-1/EK-3 cells.

**Fluorimetric measurement of [Ca\(^{2+}\)]\(_m\)**

For measurement of [Ca\(^{2+}\)]\(_m\) coverslip-attached rat glomerulosa or INS-1/EK-3 cells were loaded with 2 \muM rhod-2 acetoxymethyl ester for 30–60 min at 37°C in the appropriate culture medium. Then the cells were washed by a modified, calcium-free Krebs–Ringer's–bicarbonate–HEPES solution containing 2 mM EGTA, followed by permeabilization with 25 \mug/ml digitonin in a cytosol-like solution containing 130 mM K\(^+\), 6 mM Na\(^+\), 1 mM H\(_2\)PO\(_4\)-, 0.3 mM free Mg\(^{2+}\), 20 mM HEPES, 2 mM ADP, 2 mM pyruvate, 2 mM EGTA, 10 mM N-(2-hydroxy-ethyl)ethylenediamine-N,N',N'-triacetic acid (HEDTA), 60 mM free Ca\(^{2+}\), and 0.3 \muM cyclosporin A, pH 7.10 [25]. The permeabilizing solution was subsequently replaced with similar cytosol-like solutions containing 140, 280, 460 or 740 nM free Ca\(^{2+}\). The permeabilization allowed both the precise adjustment of cytosolic [Ca\(^{2+}\)] and the removal of any dye remaining in the cytoplasm. The free [Ca\(^{2+}\)] and [Mg\(^{2+}\)] of the solutions were adjusted by adding different amounts of CaCl\(_2\) and MgCl\(_2\) calculated by the Chelator software [28] and checked with a Ca\(^{2+}\) selective electrode (Orion, Cambridge, UK), using a Ca\(^{2+}\) calibration buffer kit (Molecular Probes) as described previously [29].

Fluorescence images were acquired using a Xenon arc lamp and an inverted microscope (Axiovert, Zeiss, Jena Germany) fitted with a 40X/1.30 oil immersion objective (FLURAR, Zeiss) and an intensified CCD camera (IC-200, PTI), under computer control. Excitation wavelengths were set by monochromators (Deltascan, PTI). Rhod-2 fluorescence was measured using an excitation wavelength of 535 nm and emission wavelength of 605 nm. Images were analyzed offline by the software ImageMaster (PTI). Up to 25 cells (selected as region of interest) were analyzed on each coverslip. Correction was applied for time-dependent reduction of fluorescence (fluorophore leakage and photobleaching), as determined from exponential fits of resting-state fluorescence. The rhod-2 signals (F) were converted to [Ca\(^{2+}\)] using the calibration equation.
Ca$^{2+}$ and mitochondrial function

Luminescence measurement of [Ca$^{2+}$]$_{im}$

Coverslip-attached INS-1/EK-3 or 143BmA-13 cells were loaded with 5 µM coelenterazine at 37°C for 2–6 h in the culture medium. The coverslips were placed in a thermostated chamber at 37°C; the cells were perfused at a rate of 1 ml/min and luminescence was measured using a photomultiplier apparatus (EMI 9789, Thorn-EMI, Middlesex, UK), as described previously [31]. Data were collected at every second on a computer photon-counting board (EMI C660). After recording the luminescence, the residual aequorin was discharged by adding 10 mM Ca$^{2+}$. The signals were expressed as a fractional rate of aequorin consumption, i.e. counts/s (L) divided by the total number of remaining counts (i.e. luminescence capacity of residual aequorin, L$_{max}$) [32]. In order to convert L/L$_{max}$ data into [Ca$^{2+}$], we developed an in situ calibration method. Permeabilized cells were exposed to the cytosol-like solution adjusted to pH 8 and completed with 1 or 5 µM FCCP and 1 or 5 µM ionomycin. Free Ca$^{2+}$ was buffered in the range of 35–630 nM, and L/L$_{max}$ was measured. [Ca$^{2+}$] could be described by a function formally similar to an equation used to describe the two-state model of aequorin [33]:

$$[\text{Ca}^{2+}] = K_0 (F_0 - F_{\text{min}})/(F_{\text{max}} - F)$$

where $K_0 = (L/L_{\text{max}})^{0.1}$. As examined in the submicromolar [Ca$^{2+}$] range, L/L$_{max}$ was hardly affected by pH in the range of 7.10–8.00, however, it was 0.5 to 1.5 log units larger (depending on [Ca$^{2+}$]) than the values predicted by the in vitro calibration curves described in the literature [34,35] (Fig. 1). The estimated concentration (luminescence evaluated with the in situ calibration) equalled with 0.996 ± nominal (electrode-measured) [Ca$^{2+}$] + 4 nM ($r = 0.996$), supporting the reliability of the in situ method. Considering that the in situ calibration relies on the physicochemical conditions of the mitochondrial matrix, moreover, the parameters of the two previously published equations have been described for cytoplasmic aequorin, we assume that the in situ calibration is more appropriate for estimating [Ca$^{2+}$]$_{im}$ in the tested submicromolar range.

Measurement of reduced pyridine nucleotides

Fluorescence of the reduced forms of the pyridine nucleotides NAD$^+$ and NADP$^+$, referred to as NAD(P)H, was performed as described for rhod-2, using excitation and emission wavelengths of 350 and 470 nm, respectively. NAD(P)H traces were normalized using the equation: normalized $F = (F_0 - F)/(F_{\text{max}} - F_0)$, where $F_0$ indicates fluorescence intensity in the control period and $F_{\text{max}}$ was measured in the presence of 10 mM β–OH butyric acid [7] plus 1 µM rotenone.

Statistics

Data are presented as means ± SEM. In fluorimetric experiments, the [Ca$^{2+}$] and NAD(P)H response of several cells on a single coverslip was averaged. Aequorin luminescence curves have been computer-averaged and the SEM of these averaged curves is also shown.

RESULTS

Measurements of mitochondrial Ca$^{2+}$ in rhod-2-loaded cells

For measurement of [Ca$^{2+}$]$_{im}$, rhod-2-preloaded rat glomerulosa and INS-1/EK-3 cells were permeabilized with digitonin in a cytosol-like solution containing 60 nM Ca$^{2+}$ for 10 min. Baseline fluorescence was recorded and solutions containing 140, 280, 460 and 740 nM free Ca$^{2+}$ were added sequentially, each for 3 min. In glomerulosa cells, mitochondria responded to [Ca$^{2+}$] elevations as
small as from 60–140 nM and further elevations of $[\text{Ca}^{2+}]_{c}$ were also followed by corresponding increases in $[\text{Ca}^{2+}]_{m}$. A representative trace (A) and statistical data (B) are shown in Figure 2. Inactivation of the $\text{Ca}^{2+}$ uniporter does not seem to limit the mitochondrial $\text{Ca}^{2+}$ response. Ruthenium red (3 µM), the inhibitor of the mitochondrial $\text{Ca}^{2+}$ uniporter, added 90 s after raising $[\text{Ca}^{2+}]_{c}$, prevented any further increase and rapidly reduced $[\text{Ca}^{2+}]_{m}$ (data not shown). The drug, added 240 s after inducing $\text{Ca}^{2+}$ uptake, terminated the plateau phase of mitochondrial $\text{Ca}^{2+}$ signal and $[\text{Ca}^{2+}]_{m}$ gradually fell towards the basal level (Fig. 3). This observation also indicates that $\text{Ca}^{2+}$ sequestration by the mitochondria was not due to a digitonin-induced increase in mitochondrial membrane permeability.

**Fig. 2** Effect of $[\text{Ca}^{2+}]_{c}$ on $[\text{Ca}^{2+}]_{m}$ in permeabilized rat glomerulosa cells. $[\text{Ca}^{2+}]_{m}$ in digitonin-permeabilized glomerulosa cells was measured with the fluorescence of rhod-2. (A) shows an original fluorimetric trace converted into $[\text{Ca}^{2+}]_{m}$. (B) shows the average $[\text{Ca}^{2+}]_{m}$ of different cell preparations, weighted with the square root of the number of coverslips per preparation. $[\text{Ca}^{2+}]_{c}$ was calculated for the last 20 s of each concentration step. Each symbol represents the average of a separate preparation.

**Fig. 3** Effect of ruthenium red on mitochondrial $\text{Ca}^{2+}$ accumulation in permeabilized rat glomerulosa cells. $[\text{Ca}^{2+}]_{m}$ was measured with the fluorescence of rhod-2. $[\text{Ca}^{2+}]_{c}$ was raised from 60 to 740 nM and 240 s later (indicated with arrow) ruthenium red (3 µM) was added. Fluorescence intensity was normalized to the prestimulation period. The mean ± SEM of 12 cells are shown.

**Fig. 4** Effect of $[\text{Ca}^{2+}]_{c}$ on $[\text{Ca}^{2+}]_{m}$ in permeabilized INS-1/EK-3 insulinoma cells. $[\text{Ca}^{2+}]_{m}$ in digitonin-permeabilized INS-1/EK-3 cells was measured with the fluorescence of rhod-2. (A) shows an original fluorimetric trace converted into $[\text{Ca}^{2+}]_{m}$. On (B), the bars show the average $[\text{Ca}^{2+}]_{m}$ of different cell preparations, weighted with the square root of the number of coverslips per preparation. $[\text{Ca}^{2+}]_{c}$ was calculated for the last 20 s of each concentration step. Each symbol represents the average of a separate preparation.
In the INS-1/EK-3 cells, 140 nM Ca\(^{2+}\) induced a slight and 280 nM Ca\(^{2+}\), a remarkable increase in three examined cell preparations. Subsequent steps to 460 and 740 nM further increased [Ca\(^{2+}\)]\(_{m}\) (Fig. 4).

Measurements of mitochondrial Ca\(^{2+}\) in mt-aequorin-expressing cells

In order to exclude unrecognized artifacts induced by the mitochondrial loading of rhod-2, we estimated the mitochondrial Ca\(^{2+}\) response also without rhod loading, by means of mitochondrially targeted aequorin (mt-aequorin). INS-1/EK-3 and the 143BmA-13 osteosarcoma cells, stably expressing mt-aequorin, were permeabilized and their mitochondrial response to [Ca\(^{2+}\)]\(_{c}\) elevations was examined in the same way as was done in the rhod-2 measurements. As shown in Figure 5 for INS-1/EK-3 cells, an increase in [Ca\(^{2+}\)]\(_{c}\) from 60 to 140 nM was followed by a large increase in [Ca\(^{2+}\)]\(_{m}\). Further steps in [Ca\(^{2+}\)]\(_{c}\) up to the examined maximum, 740 nM, induced further elevations of [Ca\(^{2+}\)]\(_{m}\). Comparable [Ca\(^{2+}\)]\(_{m}\) elevations were observed in the osteosarcoma cells, although the response to 140 nM extramitochondrial Ca\(^{2+}\) was just detectable (Fig. 6).

Effect of elevated extramitochondrial [Ca\(^{2+}\)] on mitochondrial NAD(P)H

In permeabilized glomerulosa cells, the elevation of cytoplasmic [Ca\(^{2+}\)] was followed by increased NAD(P)H fluorescence. Increased reduction of pyridine nucleotides could already be recorded when extramitochondrial [Ca\(^{2+}\)] was raised from 60 to 140 nM and a larger response was recorded at 740 nM (Fig. 7). Due to the very weak signals, we could not examine NAD(P)H formation in the INS-1/EK-3 cells.

DISCUSSION

Cytoplasmic Ca\(^{2+}\) signal, occurring in response to angiotensin II or vasopressin in rat adrenal glomerulosa
These cells are INS-1 cells, stably transfected with the response to the closure of ATP-sensitive K<sup>−/p59</sup>-sensitive luminescent photoprotein aequorin tar-
mented to the mitochondria [11]. In pancreatic producing cells [26]. The purpose of the present study was erogeneity, an increase in [Ca2<sup>+</sup>]c, already detectable at 140 nM. This observation suggests that the responsiveness to low submicromolar Ca2<sup>+</sup> is not exclusive for steroid-producing cells.

Hitherto glomerulosa and INS-1/EK-3 cells were examined using the Ca2<sup>+</sup>-sensitive dye rhod-2. In order to confirm these results, we performed these experiments without rhod-2, monitoring mt-aequorin luminescence. For a quantitative estimation of the results, we calibrated the luminescence of mitochondrial aequorin using an in situ technique. For this purpose, mitochondrial matrix [Ca2<sup>+</sup>]m was equilibrated with the extramitochondrial Ca2<sup>+</sup> buffer by means of dissipating the mitochondrial membrane potential with FCCP and accelerating Ca2<sup>+</sup> equilibration with the Ca2<sup>+</sup>-ionophore ionomycin. (The completeness of depolarization was borne out by the lack of the additional depolarizing effect of the electron-chain inhibitor rotenone, as measured fluorimetrically with tetramethyl rhodamine ethylesther (data not shown).

Using this calibration method, the calculated [Ca2<sup>+</sup>]m values were considerably lower than those calculated with formulae described for in vitro calibration in the literature [34,35]. A similar difference between the in situ and in vitro aequorin calibration curves was found in plant protoplasts [42]. The well-known discrepancy between the low and high [Ca2<sup>+</sup>]m values as obtained with rhod-2 and aequorin, respectively, has been attributed to ‘the intrinsic physicochemical characteristics of the different probes and the heterogeneity of the response within the organelle population’ [43]. Considering that in the present experiments, under otherwise identical conditions, aequorin-measured [Ca2<sup>+</sup>]m were much nearer to the rhod-2-measured values if aequorin was calibrated with in situ rather than in vitro methods [34,35], the in situ calibration seems to be preferable.

By applying mt-aequorin, we could observe a clearcut mitochondrial response to the smallest examined increase in [Ca2<sup>+</sup>]c. Raising the extramitochondrial [Ca2<sup>+</sup>]m from 60 to 140 nM gave rise to an easily detectable increase in [Ca2<sup>+</sup>]m<sub>ao</sub>. Further increases in [Ca2<sup>+</sup>]m<sub>ao</sub> were followed by further increases in [Ca2<sup>+</sup>]m. The observed responses differed from the rhod responses in two respects only. First, there was a quantitative difference in the response to the smallest elevations of [Ca2<sup>+</sup>]m<sub>ao</sub>. Observing larger responses to 140 and 280 nM Ca2<sup>+</sup> with mt-aequorin was not surprising since rhod-2 has a calcium chelating property. Therefore, the larger responses observed with mt-aequorin seem to be more correct. The second difference relates to response kinetics. Higher and
higher steady-state of \([\text{Ca}^{2+}]_{m}\) was attained or approached after each step of \([\text{Ca}^{2+}]_{m}\) elevation in the rhod-2 measurements. When \([\text{Ca}^{2+}]_{m}\) was monitored with mt-aequorin, the stepwisely increasing peak values were followed by some decrease in the calculated \([\text{Ca}^{2+}]_{m}\). In this respect it should be considered that due to the irreversible consumption of aequorin at binding \(\text{Ca}^{2+}\), its light emission may be influenced by intracellular heterogeneities in \([\text{Ca}^{2+}]\). Provided that mitochondrial heterogeneity in respect of \([\text{Ca}^{2+}]\) reflects different \(\text{Ca}^{2+}\) uptake ability, it may be assumed that aequorin in mitochondria attaining the highest \([\text{Ca}^{2+}]\) is consumed first, and then light emission is reduced. Light emission will increase again at the next \([\text{Ca}^{2+}]\) step, however, the light emission from the previously responding mitochondria will be rapidly extinguished and the second luminescence response will be dominated by a less responsive group of mitochondria. In fact, no biphasic responses were observed in the calibration measurements where the application of FCCP plus ionomycin eliminated this type of inhomogeneity of mitochondria. Computer simulation of aequorin luminescence in a multicompartment system also yielded biphasic concentration elevations (JP, unpublished data). Unfortunately, all calibration methods are based on the actual luminescing capacity of the still unoxidized aequorin content of the whole cell population \(t_{\text{max}}\), and presently no method is available for correcting for this inhomogeneity of \(\text{Ca}^{2+}\) compartments. Nevertheless, this deficiency of aequorin measurements does not influence the conclusion: low submicromolar cytoplasmic \(\text{Ca}^{2+}\) signals are transferred into the mitochondrial matrix.

We also examined the osteosarcoma 143BmA-13 cell, a cell type for which we are not aware of a cell-specific role of NAD(P)H (such as steroid hydroxylation in glomerulosa cells) or that of ATP (such as the induction of exocytosis in pancreatic \(\beta\)-cells). Measurement of \([\text{Ca}^{2+}]_{m}\) with aequorin again revealed a responsiveness to submicromolar \(\text{Ca}^{2+}\), however, the response to 140 nM \(\text{Ca}^{2+}\) was much smaller than that observed in INS-1/EK-3 cells. It follows that the mitochondrial responsiveness to submicromolar \([\text{Ca}^{2+}]_{m}\) is not exclusive either for neurons (see references in the Introduction) or for endocrine cells.

As found and discussed, submicromolar \(\text{Ca}^{2+}\) signals do induce but moderate mitochondrial \(\text{Ca}^{2+}\) signals, therefore the question may be raised whether such signals have physiological significance. To answer this question, we examined the effect of increased \([\text{Ca}^{2+}]_{m}\) on the formation of NAD(P)H in glomerulosa cells, under conditions identical to the \([\text{Ca}^{2+}]_{m}\) measurements. Cytoplasmic pyridine nucleotides should have been dialysed through the permeabilized plasma membrane. The mitochondrial origin of the signal has been established by the effect of mitochondrial substrate or drugs such as \(\beta\)-hydroxy-butyrate, FCCP and rotenone. The level of reduced pyridine nucleotides increased in function of \([\text{Ca}^{2+}]\), showing a response already to 140 nM \(\text{Ca}^{2+}\). This observation suggests that the formation of small mitochondrial \(\text{Ca}^{2+}\) signals may have a physiological bearing.

The significance of high-\(\text{Ca}^{2+}\) microdomains in the control of mitochondrial function is not in contrast with the observed effect of submicromolar \(\text{Ca}^{2+}\). The rapid formation of large mitochondrial \(\text{Ca}^{2+}\) signals during IP\(_3\)-induced \(\text{Ca}^{2+}\) release (e.g. in response to haemorrhage-induced angiotensin II formation) in glomerulosa cells or during voltage-dependent \(\text{Ca}^{2+}\) entry (e.g. after a carbohydrate-rich meal) in \(\beta\)-cells supports the immediate and efficient response of the endocrine cell to the acute stimulus. The activation of mitochondria by submicromolar \(\text{Ca}^{2+}\) signals may be important during weak but tonic stimulation of the cell (e.g. increased dietary intake of potassium or chronic sodium depletion in case of glomerulosa cells). The ability of mitochondria to sequester \(\text{Ca}^{2+}\) already in the submicromolar range may also be an important protective mechanism not only under pathological conditions (e.g. hypoxia), as hitherto assumed, but during physiological responses of the cell as well.

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