Research

Cell Calcium (2002) **31**(2), 97–104

doi: 10.1054/ceca.2001.0264, available online at http://www.idealibrary.com on IDEAL®

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. Spät[1]

[1]Department of Physiology and Laboratory of Cellular and Molecular Physiology, Semmelweis University, Faculty of Medicine, PO box 259, H-1444, Budapest, Hungary
[2]Division 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 (143BmA-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.

INTRODUCTION

'The renaissance of mitochondrial calcium transport', the title of a recently published review [1], appropriately characterizes the current interest in the role of mitochondria played in the control of intracellular calcium metabolism. It was beleived that mitochondria sequester Ca^{2+} in case of calcium intoxication but have no role in the physiological control of calcium metabolism. This view was first challenged by the description of Ca^{2+}-sensitive mitochondrial dehydrogenases [2]. Thereafter, in the last decade it was revealed that cytoplasmic Ca^{2+} signals within the physiological range increase mitochondrial matrix Ca^{2+} ($[Ca^{2+}]_m$) [3] and promote the reduced state of mitochondrial pyridine nucleotides [4–7]. The discovery of high-Ca^{2+} microdomains in the perimitochondrial cytoplasm near the inositol 1,4,5-trisphosphate (IP_3)-sensitive Ca^{2+} stores [8] provides explanation for the observed large mitochondrial Ca^{2+} signal [7,9] and NAD(P)H formation [10] evoked by IP_3 -mediated agonists. Similarly, the abrupt increase of the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_c$) in the subplasmalemmal space may account for the activation of mitochondrial metabolism in response to Ca^{2+} entry in insulin-producing INS-1 cells [11] as well as in neurons [12]. Calcium not only activates the mitochondrial substrate dehydrogenases, but conversely the rapid cycling of mitochondrial calcium may also reshape cytoplasmic Ca^{2+} signal, induced by the opening of either IP_3 receptors [7,13–16], ryanodine receptors [17] or voltage-activated Ca^{2+} channels [11,18–20]. These observations led to the general view that rapid formation of micromolar $[Ca^{2+}]$ in the perimitochondrial space is required for the activation of mitochondria (e.g. [17,21]). Certainly, this

Received 27 September 2001
Accepted 26 November 2001

Correspondence to: Dr A. Spät, Department of Physiology, Semmelweis University, Faculty of Medicine, PO box 259 H-1444 Budapest, Hungary. Tel.: 36 1 266 9180; fax: 36 1 266 6504; e-mail: spat@puskin.sote.hu

assumption is in harmony with the low affinity of the mitochondrial Ca^{2+} uniporter for Ca^{2+} ($K_d \approx 10^{-5}$ 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 NAD(P)H [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 acetoxy methylester 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 Krebs–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; 2×10^5 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 μM rhod-2 acetoxymethyl ester for 30–60 min at 37°C in the appropriate culture medium. Then the cells were washed with a modified, calcium-free Krebs–Ringer's–bicarbonate–HEPES solution containing 2 mM EGTA, followed by permeabilization with 25 μg/ml digitonin in a cytosol-like solution containing 130 mM K^+, 6 mM Na^+, 1 mM $H_2PO_4^-$, 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 nM free Ca^{2+}, and 0.3 μM 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 (FLUAR, 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

$[Ca^{2+}] = K_d(F - F_{min})/(F_{max} - F)$ [30]. F_{max} and F_{min} were determined for each cell with adding 1 μM ionomycin and 1 μM carbonyl cyanide *p*-trifluoromethoxyphenyl-hydrazone (FCCP) in the presence of 500 μM or no Ca^{2+}, respectively. A K_d value of 490 nM, determined in mitochondria exposed to FCCP and ionomycin, was applied, which is in good agreement with the value determined in vitro [19].

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

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 perifused 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]:

$$[Ca^{2+}] \text{ (in M)} = (4R - 1)/(7.1E6 * (1 - R))$$

where $R = (L/L_{max})^{0.1}$. 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 $[Ca^{2+}]_m$, 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 wavelenghts of 350 and 470 nm, respectively. NAD(P)H traces were normalized using the equation: normalized $F = (F - F_0)/(F_{max} - F_0)$, where F_0 indicates fluorescence intensity in the control period and F_{max} was measured in the presence of 10 mM β–OH butyric acid [7] plus 1 μM rotenone.

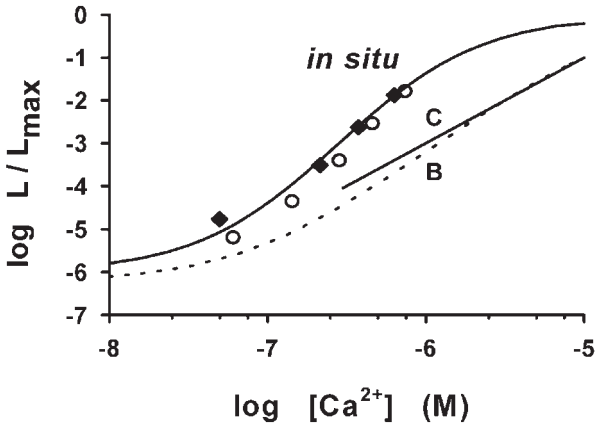

Fig. 1 Calibration of mt-aequorin. Digitonin permeabilized INS-1/EK-3 and 143BmA-13 cells were exposed to the cytosol-like solution and completed with 1 μM FCCP and 1 μM ionomycin. The lines indicated as 'in situ', C and B show normalized luminescene (L/L_{max}) values in function of extramitochondrial $[Ca^{2+}]$ as calculated with the formula described in the Materials and methods section for the applied in situ calibration, and with formulae described for in vitro methods by Cobbold and Rink [34] and Brini et al. [35], respectively. Measured L/L_{max} values are shown for pH 7.1 (empty circles, mean of duplicates) and pH 8.0 (full squares, mean of four experiments). Data on INS-1/EK-3 and 143BmA-13 cells have been pooled. (Bars of SEM are smaller than the shown symbols.)

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 $[Ca^{2+}]_m$, 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 $[Ca^{2+}]_c$ elevations as

small as from 60–140 nM and further elevations of $[Ca^{2+}]_c$ were also followed by corresponding increases in $[Ca^{2+}]_m$. A representative trace (A) and statistical data (B) are shown in Figure 2. Inactivation of the Ca^{2+} uniporter does not seem to limit the mitochondrial Ca^{2+} response. Ruthenium red (3 μM), the inhibitor of the mitochondrial Ca^{2+} uniporter, added 90 s after raising $[Ca^{2+}]_c$, prevented any further increase and rapidly reduced $[Ca^{2+}]_m$ (data not shown). The drug, added 240 s after inducing Ca^{2+} uptake, terminated the plateau phase of mitochondrial Ca^{2+} signal and $[Ca^{2+}]_m$ gradually fell towards the basal level (Fig. 3). This observation also indicates that Ca^{2+} sequestration by the mitochondria was not due to a digitonin-induced increase in mitochondrial membrane permeability.

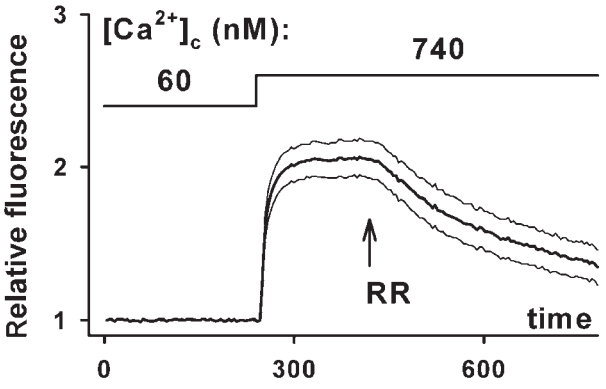

Fig. 3 Effect or ruthenium red on mitochondrial Ca^{2+} accumulation in permeabilized rat glomerulosa cells. $[Ca^{2+}]_m$ was measured with the fluorescence of rhod-2. $[Ca^{2+}]_c$ was raised from 60 to 740 nM and 240 s later (indicated with arrow) ruthenium red (3 μM) was added. Fluorescence intensity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

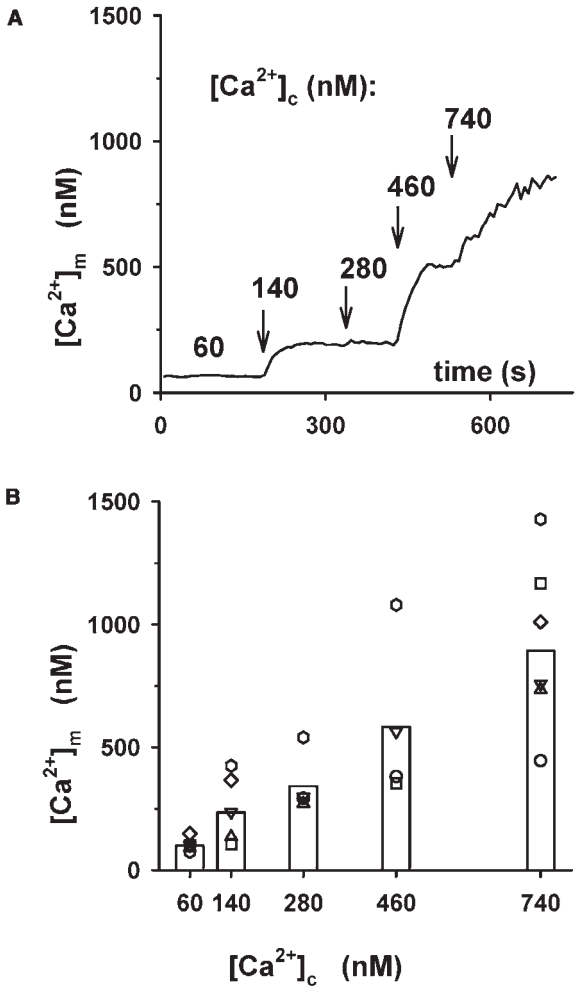

Fig. 2 Effect of $[Ca^{2+}]_c$ on $[Ca^{2+}]_m$ in permeabilized rat glomerulosa cells. $[Ca^{2+}]_m$ in digitonin-permeabilized glomerulosa cells was measured with the fluorescence of rhod-2. (A) shows an original fluorimetric trace converted into $[Ca^{2+}]$. (B) shows the average $[Ca^{2+}]_m$ of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

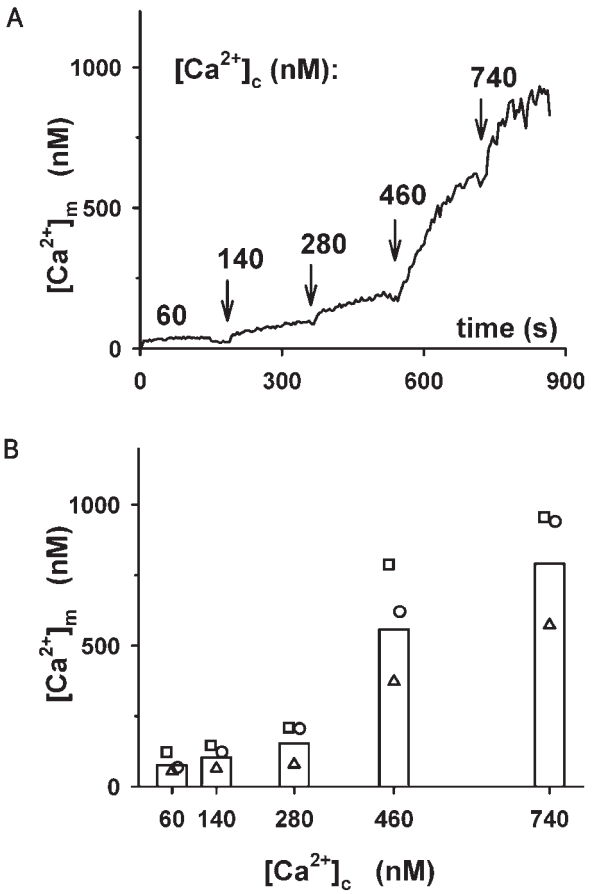

Fig. 4 Effect of $[Ca^{2+}]_c$ on $[Ca^{2+}]_m$ in permeabilized INS-1/EK-3 insulinoma cells. $[Ca^{2+}]_m$ in digitonin-permeabilized INS-1/EK-3 cells was measured with the fluorescence of rhod-2. (A) shows an original fluorimetric trace converted into $[Ca^{2+}]$. On (B), the bars show the average $[Ca^{2+}]_m$ of different cell preparations, weighted with the square root of the number of coverslips per preparation. $[Ca^{2+}]$ 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

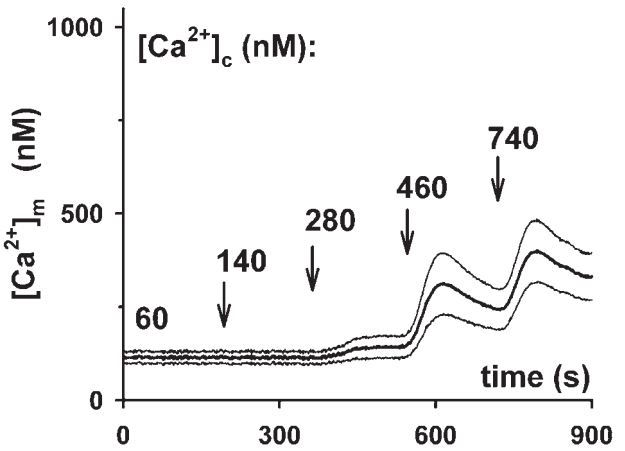

Fig. 6 Effect of extramitochondrial $[Ca^{2+}]$ on mitochondrial $[Ca^{2+}]$ in permeabilized osteosarcoma cells. $[Ca^{2+}]_m$ in digitonin-permeabilized 143BmA-13 cells was measured with the luminescence of mitochondrially targeted aequorin (mt-aequorin). The thick and the two thin lines show the mean ± SEM of seven coverslips, derived from three different cell passages.

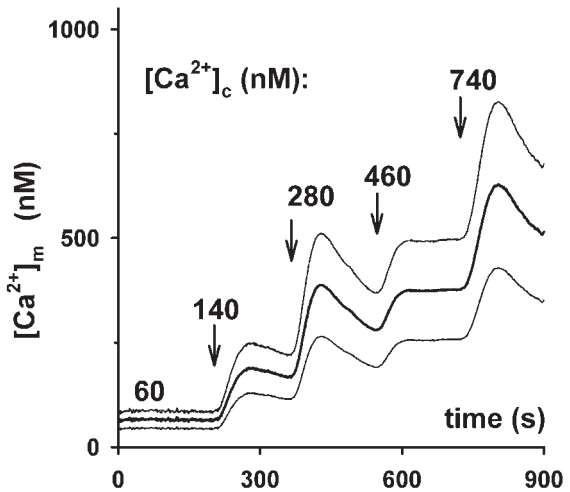

Fig. 5 Effect of $[Ca^{2+}]_c$ on $[Ca^{2+}]_m$ in permeabilized INS-1/EK-3 cells. $[Ca^{2+}]_m$ was measured with the luminescence of mitochondrially targeted aequorin (mt-aequorin). The thick and the two thin lines show the mean ± SEM of nine coverslips, respectively, derived from three different cell passages.

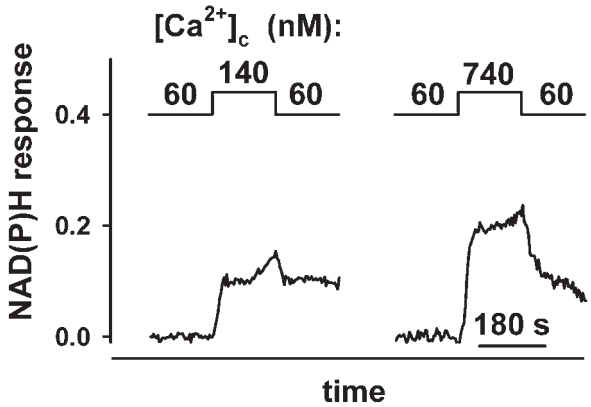

Fig. 7 Effect of extramitochondrial Ca^{2+} on mitochondrial NAD(P)H formation in permeabilized glomerulosa cells. Reduced pyridine nucleotides (NADH plus NADPH) were measured by means of their autofluorescence. The left curve shows the normalized NAD(P)H response (see Materials and methods) to raising $[Ca^{2+}]_c$ from 60 to 140 nM; the right curve shows the response to a $[Ca^{2+}]$ step to 740 nM. Each line shows the average of 5–5 coverslips, derived from the same two cell preparations.

cells [6,36] or to prostaglandin $F_{2\alpha}$ in rat luteal cells [25] stimulates the reduction of mitochondrial pyridine nucleotides. The Ca^{2+} signal, evoked by the Ca^{2+} mobilizing agonists, is brought about by IP_3-induced Ca^{2+} release [37] as well as by store-operated (capacitative) Ca^{2+} influx [25,38]. The mitochondrial Ca^{2+} signal and the ensuing activation of Ca^{2+}-dependent mitochondrial dehydrogenases [2] during the action of IP_3 may be accounted for by the transfer of the Ca^{2+} signal from the transiently formed high-Ca^{2+} microdomains between the IP_3 receptors and the vicinal mitochondria into the mitochondrial matrix [8,39]. The mechanism of activation of mitochondria during store-operated Ca^{2+} influx in glomerulosa [10] and luteal cells [25] probably differs from that during IP_3-induced Ca^{2+} release. The global Ca^{2+} signal does not exceed 300 nM and no formation of perimitochondrial high-Ca^{2+} microdomains may be expected under such conditions [3], yet mitochondrial NAD(P)H formation is enhanced. In analyzing this apparent contradiction in rhod-2-preloaded, permeabilized luteal cells, we found that a Ca^{2+} signal as small as 180 nM was transferred into the mitochondrial matrix [25]. In view of the cofactor function of NADPH in mitochondrial steroid hydroxylation and the relatively low but often sustained Ca^{2+} signals in these cell types, we assumed that the mitochondrial response to low submicromolar Ca^{2+} signals is a special characteristic of steroid-producing cells [26]. The purpose of the present study was to test whether this responsiveness is in fact specific for steroid-producing cells or is a more general phenomenon.

First we observed that the mitochondrial Ca^{2+} response to the submicromolar cytoplasmic Ca^{2+} signal is not a unique property of luteal cells but is also displayed by glomerulosa cells. Stepwise increases in $[Ca^{2+}]_c$ between 60 and 740 nM induced stepwise $[Ca^{2+}]_m$ increases in permeabilized glomerulosa cells, as measured with the Ca^{2+}-sensitive dye rhod-2. Although the response of different cell preparations showed some heterogeneity, an increase in $[Ca^{2+}]_c$ to 140 nM raised $[Ca^{2+}]_m$ in each cell preparation.

As the next step, we extended this study to a β-cell-derived, insulin-producing cell line, termed INS-1/EK-3. These cells are INS-1 cells, stably transfected with the Ca^{2+}-sensitive luminescent photoprotein aequorin targeted to the mitochondria [11]. In pancreatic β-cell and its tumour variants, exocytosis of insulin is induced by voltage-dependent Ca^{2+} influx, which in turn is a response to the closure of ATP-sensitive K^+ channels. Thus, increased formation of ATP is a prerequisite for insulin secretion and the Ca^{2+} sensitivity of mitochondria may be essential in this respect [11]. Ca^{2+}-induced acceleration of the Krebs cycle also results in enhanced production of glutamate, a recently proposed cofactor of insulin release[40]. Small $[Ca^{2+}]_m$ increases were found also to potentiate the metabolic and secretory responses of the β-cell to subsequent exposure to glucose [11,41]. Altogether, any increase in $[Ca^{2+}]_m$ may significantly modify the function of these cells. In rhod-2-preloaded, permeabilized INS-1/EK-3 cells there was a gradual increase of $[Ca^{2+}]_m$ in response to stepwise elevation of $[Ca^{2+}]_c$, already detectable at 140 nM. This observation suggests that the responsiveness to low submicromolar Ca^{2+} is not exclusive for steroid-producing cells.

Hitherto glomerulosa and INS-1/EK-3 cells were examined using the Ca^{2+}-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 $[Ca^{2+}]$ was equilibrated with the extramitochondrial Ca^{2+} buffer by means of dissipating the mitochondrial membrane potential with FCCP and accelerating Ca^{2+} equilibration with the Ca^{2+} ionophore ionomycine. (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 $[Ca^{2+}]$ 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 $[Ca^{2+}]_m$ 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 $[Ca^{2+}]_m$ 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 $[Ca^{2+}]_c$. Raising the extramitrochondrial $[Ca^{2+}]$ from 60 to 140 nM gave rise to an easily detectable increase in $[Ca^{2+}]_m$. Further increases in $[Ca^{2+}]_c$ were followed by further increases in $[Ca^{2+}]_m$. 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 $[Ca^{2+}]_c$. Observing larger responses to 140 and 280 nM Ca^{2+} 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 $[Ca^{2+}]_m$ was attained or approached after each step of $[Ca^{2+}]_c$ elevation in the rhod-2 measurements. When $[Ca^{2+}]_m$ was monitored with mt-aequorin, the stepwisely increasing peak values were followed by some decrease in the calculated $[Ca^{2+}]_m$. In this respect it should be considered that due to the irreversible consumption of aequorin at binding Ca^{2+}, its light emission may be influenced by intracellular heterogeneities in $[Ca^{2+}]$. Provided that mitochondrial heterogeneity in respect of $[Ca^{2+}]$ reflects different Ca^{2+} uptake ability, it may be assumed that aequorin in mitochondria attaining the highest $[Ca^{2+}]$ is consumed first, and then light emission is reduced. Light emission will increase again at the next $[Ca^{2+}]$ 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 (L_{max}), and presently no method is available for correcting for this inhomogeneity of Ca^{2+} compartments. Nevertheless, this deficiency of aequorin measurements does not influence the conclusion: low submicromolar cytoplasmic 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 β-cells). Measurement of $[Ca^{2+}]_m$ with aequorin again revealed a responsiveness to submicromolar Ca^{2+}, however, the response to 140 nM Ca^{2+} was much smaller than that observed in INS-1/EK-3 cells. It follows that the mitochondrial responsiveness to submicromolar $[Ca^{2+}]_c$ is not exclusive either for neurons (see references in the Introduction) or for endocrine cells.

As found and discussed, submicromolar Ca^{2+} signals do induce but moderate mitochondrial 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 $[Ca^{2+}]_c$ on the formation of NAD(P)H in glomerulosa cells, under conditions identical to the $[Ca^{2+}]_m$ 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 β-hydroxy-butyrate, FCCP and rotenone. The level of reduced pyridine nucleotides increased in function of $[Ca^{2+}]$, showing a response already to 140 nM Ca^{2+}. This observation suggests that the formation of small mitochondrial Ca^{2+} signals may have a physiological bearing.

The significance of high-Ca^{2+} microdomains in the control of mitochondrial function is not in contrast with the observed effect of submicromolar Ca^{2+}. The rapid formation of large mitochondrial Ca^{2+} signals during IP_3-induced Ca^{2+} release (e.g. in response to haemorrhage-induced angiotensin II formation) in glomerulosa cells or during voltage-dependent Ca^{2+} entry (e.g. after a carbohydrate-rich meal) in β-cells supports the immediate and efficient response of the endocrine cell to the acute stimulus. The activation of mitochondria by submicromolar 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 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.

ACKNOWLEDGEMENTS

The valuable discussions with Dr György Szabadkai and the skillful technical assistance of Ms Anikó Rajki, Ms Erzsébet Horváth and Ms Gaelle Chaffard in cell culturing is highly appreciated. This work was supported by grants from the Hungarian National Science Foundation (OTKA No. T-014649 and T-026173), the Hungarian Council for Medical Sciences (ETT No. 528/96), the Hungarian Academy of Sciences (AKP No. 96-236/53) (to AS) , the Paul Langerhans Research Fellowship from the European Association for the Study of Diabetes (to PM) and the Swiss National Science Foundation (grant No. 32-49755.96, to CBW).

REFERENCES

1. Pozzan T, Rizzuto R. The renaissance of mitochondrial calcium transport. *Eur J Biochem* 2000; **267**: 5269–5273.
2. McCormack JG, Halestrap AP, Denton RM. Role of calcium ions in regulation of mammalian intramitochondrial metabolism. *Physiol Rev* 1990; **70**: 391–425.
3. Rizzuto R, Simpson AWM, Brini M, Pozzan T. Rapid changes of mitochondrial Ca^{2+} revealed by specifically targeted recombinant aequorin. *Nature* 1992; **358**: 325–327.
4. Pralong W-F, Hunyady L, Várnai P, Wollheim CB, Spät A. Pyridine nucleotide redox state parallels production of aldosterone in potassium-stimulated adrenal glomerulosa cells. *Proc Natl Acad Sci USA* 1992; **89**: 132–136.
5. Duchen MR. Ca^{2+}-dependent changes in the mitochondrial energetics in single dissociated mouse sensory neurons. *Biochem J* 1992; **283**: 41–50.

6. Pralong W-F, Spät A, Wollheim CB. Dynamic pacing of cell metabolism by intracellular Ca^{2+}. *J Biol Chem* 1994; **269**: 27310–27314.
7. Hajnóczky G, Robb-Gaspers LD, Seitz MB, Thomas AP. Decoding of cytosolic calcium oscillations in the mitochondria. *Cell* 1995; **82**: 415–424.
8. Rizzuto R, Pinton P, Carrington W et al. Close contacts with the endoplasmic reticulum as determinants of mitochondrial Ca^{2+} responses. *Science* 1998; **280**: 1763–1766.
9. Rizzuto R, Brini M, Murgia M, Pozzan T. Microdomains with high Ca^{2+} close to IP_3-sensitive channels that are sensed by neighboring mitochondria. *Science* 1993; **262**: 744–747.
10. Rohács T, Tory K, Dobos A, Spät A. Intracellular calcium release is more efficient than calcium influx in stimulating mitochondrial NAD(P)H formation in adrenal glomerulosa cells. *Biochem J* 1997; **328**: 525–528.
11. Kennedy ED, Rizzuto R, Theler JM et al. Glucose-stimulated insulin secretion correlates with changes in mitochondrial and cytosolic Ca^{2+} in aequorin-expressing INS-1 cells. *J Clin Invest* 1996; **98**: 2524–2538.
12. Pivovarova NB, Hongpaisan J, Andrews SB, Friel DD. Depolarization-induced mitochondrial Ca accumulation in sympathetic neurons: Spatial and temporal characteristics. *J Neurosci* 1999; **19**: 6372–6384.
13. Duchen MR. Contributions of mitochondria to animal physiology: from homeostatic sensor to calcium signalling and cell death. *J Physiol (Lond)* 1999; **516**: 1–17.
14. Tinel H, Cancela JM, Mogami H et al. Active mitochondria surrounding the pancreatic acinar granule region prevent spreading of inositol trisphosphate-evoked local cytosolic Ca^{2+} signals. *EMBO J* 1999; **18**: 4999–5008.
15. Kaftan EJ, Xu T, Abercrombie RF, Hille B. Mitochondria shape hormonally induced cytoplasmic calcium oscillations and modulate exocytosis. *J Biol Chem* 2000; **275**: 25465–25470.
16. Sherer TB, Trimmer PA, Parks JK, Tuttle JB. Mitochondrial DNA-depleted neuroblastoma (Rho°) cells exhibit altered calcium signaling. *Biochim Biophys Acta Mol Cell Res* 2000; **1496**: 341–355.
17. Szalai G, Csordás G, Hantash BM, Thomas AP, Hajnóczky G. Calcium signal transmission between ryanodine receptors and mitochondria. *J Biol Chem* 2000; **275**: 15305–15313.
18. Thayer SA, Miller RJ. Regulation of the intracellular free calcium concentration in single rat dorsal root ganglion neurones in vitro. *J Physiol (Lond)* 1990; **425**: 85–115.
19. Babcock DF, Herrington J, Goodwin PC, Park YB, Hille B. Mitochondrial participation in the intracellular Ca^{2+} network. *J Cell Biol* 1997; **136**: 833–844.
20. David G. Mitochondrial clearance of cytosolic Ca^{2+} in stimulated lizard motor nerve terminals proceeds without progressive elevation of mitochondrial matrix $[Ca^{2+}]$. *J Neurosci* 1999; **19**: 7495–7506.
21. Montero M, Alonso MT, Carnicero E et al. Chromaffin-cell stimulation triggers fast millimolar mitochondrial Ca^{2+} transients that modulate secretion. *Nat Cell Biol* 2000; **2**: 57–61.
22. Gunter TE, Pfeiffer DR. Mechanisms by which mitochondria transport calcium. *Am J Physiol* 1990; **258**: C755-C786.
23. Friel DD, Tsien RW. An FCCP-sensitive Ca^{2+} store in bullfrog sympathetic neurons and its participation in stimulus-evoked changes in $[Ca^{2+}]_i$. *J Neurosci* 1994; **14**: 4007–4024.
24. Colegrove SL, Albrecht MA, Friel DD. Dissection of mitochondrial Ca^{2+} uptake and release fluxes in situ after depolarization-evoked $[Ca^{2+}]_i$ elevations in sympathetic neurons. *J Gen Physiol* 2000; **115**: 351–369.
25. Szabadkai G, Pitter JG, Spät A. Cytoplasmic Ca^{2+} at low submicromolar concentration stimulates mitochondrial metabolism in rat luteal cells. *Pflügers Arch* 2001; **441**: 678–685.
26. Spät A, Pitter JG, Rohács T, Szabadkai Gy. Stimulus-secretion coupling and mitochondrial metabolism in steroid secreting cells. *News Physiol Sci* 2001; **16**: 197–200.
27. Spät A, Balla I, Balla T, Cragoe EJ Jr, Hajnóczky Gy, Hunyady L. Angiotensin II and potassium activate different calcium entry mechanisms in rat adrenal glomerulosa cells. *J Endocrinol* 1989; **122**: 361–370.
28. Schoenmakers TJ, Visser GJ, Flik G, Theuvenet AP. CHELATOR: an improved method for computing metal ion concentrations in physiological solutions. *Biotechniques* 1992; **12**: 870–876.
29. Maechler P, Kennedy ED, Pozzan T, Wollheim CB. Mitochondrial activation directly triggers the exocytosis of insulin in permeabilized pancreatic β-cells. *EMBO J* 1997; **16**: 3833–3841.
30. Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* 1985; **260**: 3440–3450.
31. Maechler P, Kennedy ED, Wang HY, Wollheim CB. Desensitization of mitochondrial Ca^{2+} and insulin secretion responses in the beta cell. *J Biol Chem* 1998; **273**: 20770–20778.
32. Cobbold PH. Cytoplasmic free calcium and amoeboid movement. *Nature* 1980; **285**: 441–446.
33. Allen DG, Blinks JR, Prendergast FG. Aequorin luminescence: relation of light emission to calcium concentration–a calcium-independent component. *Science* 1977; **195**: 996–998.
34. Cobbold PH, Rink TJ. Fluorescence and bioluminescence measurement of cytoplasmic free calcium. *Biochem J* 1987; **248**: 313–328.
35. Brini M, Marsault R, Bastianutto C, Alvarez J, Pozzan T, Rizzuto R. Transfected aequorin in the measurement of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$). A critical evaluation. *J Biol Chem* 1995; **270**: 9896–9903.
36. Rohács T, Nagy Gy, Spät A. Cytoplasmic Ca^{2+} signalling and reduction of mitochondrial pyridine nucleotides in adrenal glomerulosa cells in response to K^+, angiotensin II and vasopressin. *Biochem J* 1997; **322**: 785–792.
37. Spät A, Enyedi P, Hajnóczky Gy, Hunyady L. Generation and role of calcium signal in adrenal glomerulosa cells. *Exp Physiol* 1991; **76**: 859–885.
38. Rohács T, Bagó A, Deák F, Hunyady L, Spät A. Capacitative Ca^{2+} influx in adrenal glomerulosa cells. Possible role in angiotensin II response. *Am J Physiol Cell Physiol* 1994; **267**: C1246–C1252.
39. Hajnóczky G, Csordás G, Krishnamurthy R, Szalai G. Mitochondrial calcium signaling driven by the IP_3 receptor. *J Bioenerg Biomembr* 2001; **32**: 15–25.
40. Maechler P, Wollheim CB. Mitochondrial glutamate acts as a messenger in glucose-induced insulin exocytosis. *Nature* 1999; **402**: 685–689.
41. Ainscow EK, Rutter GA. Mitochondrial priming modifies Ca^{2+} oscillations and insulin secretion in pancreatic islets. *Biochem J* 2001; **353**: 175–180.
42. Gilroy S, Hughes WA, Trewavas AJ. A comparison between quin-2 and aequorin as indicators of cytoplasmic calcium levels in higher plant cell protoplasts. *Plant Physiol* 1989; **90**: 482–491.
43. Rizzuto R, Bernardi P, Pozzan T. Mitochondria as all-round players of the calcium game. *J Physiol (Lond)* 2000; **529**: 37–47.