Simultaneous Measurements of Cytosolic and Mitochondrial Ca\(^{2+}\) Transients in HT29 Cells*

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‡The abbreviations used are: FDIM, fluorescence digital imaging; DiOC\(_{18}\)(3), 3,3′-dipropylthiocarbocyanine iodide; [Ca\(^{1+}\)]\(_i\), intracellular free Ca\(^{2+}\) activity; [Ca\(^{2+}\)]\(_m\), mitochondrial free Ca\(^{2+}\) activity; [Ca\(^{2+}\)]\(_c\), cytosolic free Ca\(^{2+}\) activity; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; CCH, carboxyatractylate; DIC, differential interference contrast; OGD, Oregon green BAPTA-1 dextran.

Loading of HT\(_{29}\) cells with the Ca\(^{2+}\) dye fura-2/AM resulted in an nonhomogeneous intracellular distribution of the dye. Cellular compartments with high fura-2 concentrations were identified by correlation with mitochondrial markers, cellular autofluorescence induced by UV, and dynamic measurement of autofluorescence after inhibition of oxidative phosphorylation. Stimulation with carbachol (10\(^{-4}\) mol/liter) increased cytosolic, nuclear, and mitochondrial Ca\(^{2+}\) activity ([Ca\(^{2+}\)]\(_c\), [Ca\(^{2+}\)]\(_n\), and [Ca\(^{2+}\)]\(_m\), respectively) measured by UV confocal and conventional imaging. Similar results were obtained with a prototype two-photon microscope (Zeiss, Jena, Germany) allowing for fura-2 excitation. The increase of [Ca\(^{2+}\)]\(_m\) lagged behind that of [Ca\(^{2+}\)]\(_c\) and [Ca\(^{2+}\)]\(_n\) by 10–20 s, and after removing the agonist, [Ca\(^{2+}\)]\(_m\) also decreased with a delay. A strong increase of [Ca\(^{2+}\)]\(_m\) occurred only when a certain threshold of [Ca\(^{2+}\)]\(_c\) (around 1 \(\mu\)mol/liter) was exceeded. In a similar way, ATP, neurotensin, and thapsigargin increased [Ca\(^{2+}\)]\(_m\). Carbonyl cyanide p-trifluoromethoxyphenylhydrazone reversibly reduced the increase of [Ca\(^{2+}\)]\(_m\). The source of the mitochondrial Ca\(^{2+}\) increase had intra- and extracellular components, as revealed by experiments in low extracellular Ca\(^{2+}\). We conclude that agonist-induced Ca\(^{2+}\) signals are transduced into mitochondria. 1) Mitochondria could serve as a Ca\(^{2+}\) sink, 2) mitochondria could allow the modulation of [Ca\(^{2+}\)]\(_c\) and [Ca\(^{2+}\)]\(_n\) signals, and 3) [Ca\(^{2+}\)]\(_m\) may serve as a stimulatory metabolic signal when a cell is highly stimulated.

Initial observations with the AM form of the Ca\(^{2+}\)-sensitive dye quin-2 indicated that the free acid form of the dye does not accumulate within intracellular compartments (1). However, using microscopic methods with higher resolution, in numerous labeling experiments with mitochondria-specific fluorescent markers, it is now well accepted that most AM dyes will accumulate within intracellular compartments. However, dye quin-2 indicated that the free acid form of the dye does not serve as a Ca\(^{2+}\) transducer into mitochondria. 1) Mitochondria could increase [Ca\(^{2+}\)]\(_c\) in the same way, ATP, neurotensin, and thapsigargin increased [Ca\(^{2+}\)]\(_m\) (21–23) previously. Experiments were performed with three different experimental set-ups. 2) Confocal Microscope—The set-up consisted of an inverted fluorescence microscope equipped with a \(\times\) 100 objective (Axiovert 100 TV plus an OPTAVAR \(\times\) 1.6 lens, Fluor 100/1.3 oil, Zeiss), a fast switching monochromator (T.I.L.L. Photonics, Planegg, Germany) for changing the excitation wavelength, and a GEN3, intensified CCD camera (ICCD 350, VideoScope, Sterling, VA) for fluorescence imaging. Images were acquired using an average of 8–16 frames to increase the signal/noise ratio. Control of the experiment, image acquisition, and data analysis were done with the software package MetaFluo/MetaMorph (Universal Imaging, West Chester, PA).

MATERIALS AND METHODS

Cell Culture and Measurements of [Ca\(^{2+}\)]\(_c\)—HT\(_{29}\) cells were cultured and prepared for measurements of [Ca\(^{2+}\)]\(_c\), with fura-2 as described (21–23) previously. Experiments were performed with three different experimental set-ups.

Camera-based System—The set-up consisted of an inverted fluorescence microscope equipped with a \(\times\) 100 objective (Axiovert 100 TV plus an OPTAVAR \(\times\) 1.6 lens, Fluor 100/1.3 oil, Zeiss), a fast switching monochromator (T.I.L.L. Photonics, Planegg, Germany) for changing the excitation wavelength, and a GEN3, intensified CCD camera (ICCD 350, VideoScope, Sterling, VA) for fluorescence imaging. Images were acquired using an average of 8–16 frames to increase the signal/noise ratio. Control of the experiment, image acquisition, and data analysis were done with the software package MetaFluo/MetaMorph (Universal Imaging, West Chester, PA). The source of the mitochondrial Ca\(^{2+}\) increase had intra- and extracellular components, as revealed by experiments in low extracellular Ca\(^{2+}\). We conclude that agonist-induced Ca\(^{2+}\) signals are transduced into mitochondria. 1) Mitochondria could serve as a Ca\(^{2+}\) sink, 2) mitochondria could allow the modulation of [Ca\(^{2+}\)]\(_c\) and [Ca\(^{2+}\)]\(_n\) signals, and 3) [Ca\(^{2+}\)]\(_m\) may serve as a stimulatory metabolic signal when a cell is highly stimulated.
were then recorded at a scanning speed of 2 or 4 s per full-size image, with a two-frame average. The fluorescence signal at 488 nm excitation consisted of more than 95% from the mitochondrial markers, the rest being cellular autofluorescence and the fura-2 emission signal.

Two-photon Microscope—A prototype microscope system (LSM 560, Zeiss) with a turn-key fiber laser (850 nm; pulse-width, <180 fs; maximum power, 7.4 mW) enabled us to image fura-2-loaded cells with a ×40 objective (Zeiss, Plan-Neofluar 40/1.3 oil). A timed series of fluorescence images (~100 images of 512 × 512 pixels; 2–4-μs pixel time; measured mean laser power at the objective plane reduced by gray filters, 1.2 mW) was recorded before and during agonist stimulation. All fluorescence emission below 680 nm was collected without using a pinhole in front of the fluorescence detector. The two-photon excitation wavelength of 780 nm should result in an excitation above the isosbestic point of fura-2. Therefore, one expects a decrease of the fluorescence signal with increasing [Ca$^{2+}$].

Calibration of the fura-2 signal in terms of [Ca$^{2+}$]$_{i}$ was performed at the end of the experiments using ionomycin as described (24). The problems of the calibration are discussed below. The average of the pixel-by-pixel ratios of user-selected areas marked inside each cell was used to calculate [Ca$^{2+}$]$_{i}$, according to Grynkiewicz et al. (25). No calibrations were performed in the experiment with the confocal or the two-photon system.

Microinjection of Oregon Green BAPTA-1 Dextran 10000—Electrodes for microinjection were pulled with a laser puller (P-2000, Sutter Instruments, Novato, CA) and the dye filled pipettes were beveled to a resistance of 20–30 MΩ using a microbeveler (BV10E, Sutter Instruments). Injections were performed by using a piezo stepper (PMZ 20, Frankenberger, Gilching, Germany) and by pressure injection of the dye (Injector 5242, Eppendorf, Hamburg, Germany). The injection solution contained 1 mmol/liter of the fluorescent dye.

Solutions and Chemicals—The standard solution contained 145 mmol/liter NaCl, 1.6 mmol/liter K$_{2}$HPO$_{4}$, 0.4 mmol/liter KH$_{2}$PO$_{4}$, 1.3 mmol/liter calcium gluconate, 1 mmol/liter MgCl$_{2}$, 5 mmol/liter β-glucose, pH 7.4. The detailed composition of the solutions used in the experiments was described recently (21). The injection solution contained 30 mmol/liter KCl, 4.8 mmol/liter Na$_{2}$HPO$_{4}$, 1.2 mmol/liter NaH$_{2}$PO$_{4}$, 95 mmol/liter potassium gluconate, 1 mmol/liter MgCl$_{2}$, 5 mmol/liter β-glucose, 0.5 mmol/liter EGTA, pH 7.2. All fluorescent dyes were from Molecular Probes (Eugene, OR). All other chemicals were of the highest grade of purity available and were obtained from Sigma or Merck (Darmstadt, Germany).

Statistics—Data are given as mean values ± S.E. (n), where n refers to the number of experiments. Paired and unpaired t tests were used to compare mean values within one experimental series and different experimental series, respectively. A P value of ≤ 0.05 was accepted to indicate statistical significance.

RESULTS

Intracellular Distribution of Fura-2 after Loading with Fura-2/AM—Loading of HT$_{29}$ cells (4–7 days after seeding) with fura-2/AM at room temperature resulted in an uneven distribution of the dye inside the cell. Fig. 1A shows a typical confocal image of such cells taken at 364 nm excitation, which is near the isosbestic point of the fura-2 spectrum. Images taken at 351 nm excitation show a similar dye distribution. Varying the loading conditions did not alter the general distribution pattern of tubular structures preferentially located around the nucleus. In younger cells (2–3 days after seeding), the perinuclear tubular structures were not as marked and showed a more punctuate distribution of the spots of high fluorescence.

In earlier reports, it was described that fluorescent dyes, such as fura-2 or fluo-3, when loaded in the AM form, accumulated in the mitochondria (4, 26, 27). Therefore, we compared the fluorescence distribution pattern of the fura-2/AM-loaded HT$_{29}$ cells after co-staining with the mitochondrial markers DiOC$_{3}(3)$ or rhodamine 6G. Fig. 1A and B, shows that the mitochondrial stain (here DiOC$_{3}(3)$) widely overlaps with the cellular structures that have accumulated high concentrations of fura-2. The differential interference contrast (DIC) image of the same area as depicted in Fig. 1C shows that the majority of the mitochondria in HT$_{29}$ cells are located around the nucleus. To prove the specificity of the used mitochondrial marker, we compared the pattern of the UV autofluorescence of HT$_{29}$ cells with the stain pattern obtained by DiOC$_{3}(3)$. In most cells, cellular autofluorescence at 364 nm is related to NADH and NADPH fluorescence mainly located in mitochondria (28). Fig. 1D and E, shows a high degree of co-localization of both fluorescence signals, and again, most of the mitochondria are located around the nucleus (Fig. 1F). Notably, when fura-2 was loaded in the non-AM form via a patch pipette or microinjected, we did not observe dye accumulation in the mitochondria (both types of experiments, n = 3; data not shown).

To further characterize the mechanism of dye accumulation,
structure (right image), whereas other surrounding mitochondria did not lose their fluorescence, indicating noncontinuous mitochondria in HT29 cells. The right image, taken 5 min after bleaching, showed no recovery within the bleached area.

we used the spot bleach mode of the confocal microscope, which allows fast wavelength and intensity changes from one scanned image to another by using acousto-optical tunable filters. Fura-2 was equilibrated for a period of 15 min at 37°C and then bleached with a short pulse (0.2 s) of high laser intensity in the central, nuclear part of the cell. This resulted in a strong decrease of the fluorescence in the cytosol as well as in the nucleus. The remaining fluorescence was stable in the mitochondria for at least 20–25 min (see Fig. 2A). In addition to the above results, bleaching of a single mitochondrion with the spot bleach method (0.02 s) after fura-2/AM loading and an equilibration period of 15 min at 37°C (to allow for the cleavage of the AM-ester) did not give a significant recovery of the fura-2 fluorescence signal (Fig. 2B). These results indicate that fura-2/AM is converted inside the mitochondrion to the fura-2 free acid, which is then trapped inside the organelle. Localized excitation spectra of fura-2 (excitation, 320–400 nm; emission, >470 nm), recorded with the fast monochromator by FDIM from single cells, showed no significant excitation shift between the three compartments (cytosol, nucleus, and mitochondria; data not shown; n = 3). Localized emission measurements, using the confocal microscope and a CCD-based spectrometer, showed no difference in the emission spectra in the nucleus and the cytosol. Emission spectra from single mitochondria could not be measured due to the limited sensitivity of the spectrometer.

Effect of Agonist Stimulation on Mitochondrial Ca2+ Activity ([Ca2+]m)—Fig. 3 shows that upon agonist stimulation, a differential intracellular Ca2+ signal was observed by confocal imaging of the fura-2 fluorescence. The top three images show again the accumulation of fura-2 in discrete organelles identified (see Fig. 1) mostly as mitochondria. The fura-2 emission signal is high, but almost equal at both excitation wavelengths. This results in a mitochondrial Ca2+ activity ([Ca2+]m) very similar to that of the cytosol or the nucleus, as shown in the first 351/364 nm ratio image on the second row of Fig. 3. Upon stimulation with a low concentration of an agonist (here, CCH at 10-6 mol/liter), [Ca2+]i rises in the cytosol, as well as in the nucleus, but only to a minor extent in the area of the mitochondria (Fig. 3, second image of the second row). However, increasing the agonist concentration (10-4 mol/liter) led to a [Ca2+]i response also in the mitochondrial area (Fig. 3, second image of the third row). A similar concentration dependent [Ca2+]m response was observed for other inositol 1,4,5-trisphosphate-coupled agonists, such as ATP and neurotensin, and is examined in more detail under Agonist Dependence and Time Course of the Mitochondrial Ca2+ Increase.

In order to address the problem of out of focus fluorescence and the contribution of cytosolic dye signals to mitochondrial dye signals we used three experimental approaches.

1) Cells were loaded with fura-2 as described under “Materials and Methods” and then, in addition, microinjected with the high molecular weight Ca2+ indicator dye Oregon green BAPTA-1 dextran (OGD) 10,000. Fig. 4A shows the typical fura-2 fluorescence image of a cell, and Fig. 4B shows the OGD fluorescence image from the same cell. It is obvious from the wide overlap of the darker intracellular structures in the OGD image with the bright structures in the fura-2 image that OGD did not enter the mitochondria. In addition, other intracellular structures, such as the nuclear envelope, show within the limits of optical resolution only a very low dye signal. To obtain a good overlap of the two images, the focus of the microscope had to be changed by around 0.4 μm, in order to compensate for a chromatic aberration of the microscope objective. Stimulation of the double-loaded cells with neurotensin or CCH showed a response only from OGD at 488 nm excitation, but almost no response in the fura-2 signals at 351 and 364 nm (data not shown, n = 5). This could be due to the great overlap of the fura-2 emission signal with the OGD absorption spectrum. However, we did not examine this in more detail. The very weak OGD signal from mitochondrial areas, which we interpret as a spill over from the cytosol, showed small intensity changes (with a poor signal:noise ratio), synchronous with the cytosolic or nuclear signal. From the optical properties of a confocal microscope, it is more difficult to exclude the surrounding bright cytosolic and nuclear signal and resolve the small dark
mitochondrial areas. Due to the focal shift, the limited z resolution, and the poorly responsive fura-2 signal, the measurements could not be used further.

2) In a second experimental approach, we tried to reduce the contribution of nonmitochondrial fluorescence to the mitochondrial signal by using a prototype two-photon microscope system for single wavelength excitation of fura-2. We acquired simultaneously the fluorescence images and transmitted light DIC images. This enabled the detection of changes in the focal plane due to movements of the preparation or movements of intracellular organelles. Two-photon excitation gives a high z resolution (0.4–0.6 μm, full-width, half-maximum) for fluorescence imaging. Fig. 5A shows a typical two-photon fluorescence image of a single cell. Three cellular areas (mitochondrion, nucleus, and cytosol) are marked inside the cell. Fig. 5B shows the fluorescence intensity changes in response to a stimulation with ATP and CCH. The fluorescence signal dropped reversibly in all compartments, with the mitochondrial area clearly delayed compared with the signal change in the other compartments. This type of experiment was successfully repeated four times.
times. In other experiments, the distinct mitochondrial structures disappeared, probably due to changes in the focal plane due to movements of the whole preparation or to fast movements of the mitochondria in the z image plane. The drop in the fura-2 signal in response to a [Ca$^{2+}$]$_{i}$ increase indicated that with a 780-nm two-photon excitation, fura-2 is excited at a wavelength longer than the isosbestic point.

3) A mitochondrial uncoupler, which would breakdown the mitochondrial potential, was used in an attempt to prevent the [Ca$^{2+}$]$_{i}$ increase. A typical experiment, using high resolution FDIM, is shown in Fig. 6A. FCCP reversibly inhibited around 50% of the Ca$^{2+}$ change in mitochondria. Fig. 6B shows a summary of the experimental series with FCCP ($n = 7$); the mean inhibition was 66.7 ± 3.6%. We could also separate the time courses of the mitochondrial Ca$^{2+}$ increase from the Ca$^{2+}$ increase in the cytosol and the nucleus by applying FCCP shortly before and during the onset of a CCH-induced Ca$^{2+}$ increase as shown in a typical experiment in Fig. 7 (one out of four similar experiments).

**Agonist Dependence and Time Course of the Mitochondrial Ca$^{2+}$ Increase**—In one series of experiments, we examined in more detail the agonist concentration dependence of the mitochondrial [Ca$^{2+}$]$_{i}$ increase. We recorded a concentration response curve for [Ca$^{2+}$]$_{c}$, [Ca$^{2+}$]$_{i}$, and [Ca$^{2+}$]$_{m}$ with carbachol, which stimulates M$_{3}$ receptors in HT$_{29}$ cells (29). Carbachol was chosen because its [Ca$^{2+}$]$_{i}$ response is only slightly desensitizing in comparison to neurotensin or ATP, as we have shown previously (22, 30). Fig. 8A shows a typical measurement of [Ca$^{2+}$]$_{i}$ in the cytosol, nucleus, and a single mitochondrial in response to different carbachol concentrations (0.5–100 μmol/liter). At low CCH concentrations, only a small increase of [Ca$^{2+}$]$_{i}$ could be measured, and it stayed well below the value of [Ca$^{2+}$]$_{i}$, and [Ca$^{2+}$]$_{m}$. At a higher agonist concentration, a biphasic increase of [Ca$^{2+}$]$_{i}$ was observed (Fig. 8A). This mitochondrial transient was delayed in the second phase, but in principle it followed [Ca$^{2+}$]$_{i}$, and [Ca$^{2+}$]$_{m}$. The peak response was slightly lower than [Ca$^{2+}$]$_{i}$, and [Ca$^{2+}$]$_{m}$. Removal of the agonist led to a delayed decrease of [Ca$^{2+}$]$_{i}$, which then stayed, in around 75% of the experiments, for 1–3 min above the [Ca$^{2+}$]$_{i}$ and [Ca$^{2+}$]$_{m}$ (see Fig. 8A (CCH, 10$^{-5}$ mol/liter) or Fig. 6A). Fig. 8B summarizes the CCH concentration response curve from 18 paired experiments. The response of [Ca$^{2+}$]$_{i}$ was significantly shifted to the right, with a 50% effect at around 0.3 μmol/liter, whereas the responses for the cytosol and the nucleus were not significantly different up to a CCH concentration of 10$^{-5}$ mol/liter. In Fig. 10A, the relationship is plotted between [Ca$^{2+}$]$_{i}$, and [Ca$^{2+}$]$_{m}$ from the experiments of the concentration response curve for CCH. It is obvious that up to a cytosolic 345/380 ratio of around 4–5 only the small, fast changes of [Ca$^{2+}$]$_{i}$ occurred. When this threshold was exceeded, a sudden increase of [Ca$^{2+}$]$_{i}$ was measured, and for higher cytosolic ratios, the mitochondrial ratio followed more directly [Ca$^{2+}$]$_{i}$, and [Ca$^{2+}$]$_{m}$. Using the fura-2 calibration values for the cytosol or the nucleus a ratio of 5 corresponds to Ca$^{2+}$ activities of approximately 1200 nmol/liter.

**Source of Ca$^{2+}$ for the Mitochondrial Ca$^{2+}$ Increase**—We further examined the mechanism of the [Ca$^{2+}$]$_{i}$ increase by using thapsigargin, which inhibits the SERCA type Ca$^{2+}$-ATPase of the intracellular Ca$^{2+}$ stores and thus increases [Ca$^{2+}$]$_{i}$, by emptying inositol 1,4,5-trisphosphate-sensitive Ca$^{2+}$ stores. Thapsigargin, as shown in Fig. 9, led to a concentration-dependent, relatively slow increase of [Ca$^{2+}$]$_{i}$, [Ca$^{2+}$]$_{m}$, and [Ca$^{2+}$]$_{n}$, (n = 7), very similar to what was measured with CCH. Again, at a cytosolic 345/380 ratio of around 4, a sharp increase of [Ca$^{2+}$]$_{m}$ was observed. Plotting the data for thapsigargin in the same way as outlined for Fig. 8A resulted in a very similar relationship between the cytosolic and the mitochondrial fura-2 ratios. This is shown in Fig. 10B. Again, the mean [Ca$^{2+}$]$_{i}$ value, at which a the second, strong and fast increase of [Ca$^{2+}$]$_{m}$ also occurred, was a ratio of around 5.

Fig. 11 shows the increase of [Ca$^{2+}$]$_{i}$ induced by neurotensin, when no Ca$^{2+}$ was present in the extracellular solution. Thus, after emptying of the neurotensin-sensitive Ca$^{2+}$ stores, no Ca$^{2+}$ influx could occur. Even then, a transient increase of [Ca$^{2+}$]$_{m}$ was measured, which implies that it must have been due to release of Ca$^{2+}$ from intracellular stores. Fig. 12 shows the effect of removal of Ca$^{2+}$ from the external medium in the plateau phase of a Ca$^{2+}$ increase, when [Ca$^{2+}$]$_{i}$, and [Ca$^{2+}$]$_{m}$ are dependent on the balance between Ca$^{2+}$ influx and Ca$^{2+}$ efflux. Removal of external Ca$^{2+}$ in this phase of the transient led to a fast decrease of whole cell [Ca$^{2+}$]$_{i}$, including [Ca$^{2+}$]$_{m}$, [Ca$^{2+}$]$_{m}$, and [Ca$^{2+}$]$_{n}$. However, it was obvious in these experiments that the time course for the decrease was slower for [Ca$^{2+}$]$_{i}$, and [Ca$^{2+}$]$_{m}$ did not decrease to the low values of [Ca$^{2+}$]$_{i}$, or [Ca$^{2+}$]$_{m}$, within the 2 min of the low Ca$^{2+}$ solution in the bath. Readdition of a solution with normal Ca$^{2+}$ (1.3 mmol/liter), still in the presence of the agonist, led to an increase of [Ca$^{2+}$]$_{i}$ in all three cellular compartments with a similar sequence, time course, and shape compared with an initial agonist stimulation. After removal of CCH (100 μmol/liter), [Ca$^{2+}$]$_{i}$ remained elevated in most experiments for more than 2 min (Figs. 8A, 12). A similar observation could be made with high concentrations of other agonists, such as ATP or neurotensin (each n = 3; data not shown). From Figs. 11 and
12, one can conclude that the \([\text{Ca}^{2+}]_m\) transient has, similar to the increase of \([\text{Ca}^{2+}]_c\) and \([\text{Ca}^{2+}]_n\), first an intracellular and then an extracellular source.

Influence of Mitochondrial \([\text{Ca}^{2+}]\) Increase on the Energy State of Mitochondria—To show the influence of agonist stimulation on the mitochondrial energy state, we measured cellular autofluorescence at 340 nm excitation and emission \(500\) nm, which, as outlined in the first paragraph under “Results,” is mostly due to the mitochondrial NAD(P)H levels. As shown in Fig. 13, treatment of the cells with KCN and NaN\(_3\) to inhibit oxidative phosphorylation and thus increase the level of NAD(P)H led to an increase of overall cellular autofluorescence. This effect was reversible and repeatable. A subsequent, strong stimulation of the cells with a maximal dose of CCH and ATP (both \(10^{-4}\) mol/liter), which will result in a strong increase of \([\text{Ca}^{2+}]_m\), as well as \([\text{Ca}^{2+}]_c\) and \([\text{Ca}^{2+}]_n\), also led to a significant increase in NAD(P)H, as indicated by the increase of autofluorescence.

DISCUSSION

The present experiments were performed to evaluate the role of mitochondrial \([\text{Ca}^{2+}]\) in response to agonist stimulation. By using confocal fura-2 imaging, two-photon microscopy, and high resolution FDIM, simultaneous measurement of the \([\text{Ca}^{2+}]\) signals in at least three cellular compartments (cytosol, nucleus, and mitochondria) was possible.
Mitochondrial Localization of Fura-2—Mitochondrial localization of a substantial amount of fura-2 after AM loading was evaluated by correlating the fura-2 distribution with the UV autofluorescence and the pattern of mitochondrial markers. Our results are comparable with reports on the fura-2 or fluo-3 distribution in myocytes (12, 14), endothelial cells (31), or hepatocytes (17). In addition, the bleaching experiments using confocal microscopy indicate that fura-2 enters the mitochondria in the AM form, where it is trapped or bound after cleavage. In addition, the bleaching of single mitochondrial structures reveals that in HT29 cells mitochondria do not form a continuous network throughout the cytosol. This is in contrast to recent findings in HeLa cells, where recombinant green fluorescent protein was used to label mitochondria (32). However, measurements made in our laboratory on primary cultures of hepatocytes also support the idea that mitochondria in these cells indeed can form a network extending over 10–20 μm.

Are the Properties of Intramitochondrial Fura-2 Comparable? S. Ricken, R. Greger, and R. Nitschke, unpublished data.

**FIG. 8.** A, typical FDIM experiment showing the dose dependent increase of the Ca$^{2+}$ activity after stimulation with different CCH concentrations in cellular areas identified as cytosol, nucleus, and a single mitochondrion. B, concentration response curve for the CCH-induced increase in Ca$^{2+}$ activity in cytosol, nucleus, and mitochondria.

**FIG. 9.** Typical FDIM experiment showing the effect of thapsigargin (20 nmol/liter) on the single cell Ca$^{2+}$ activity in cellular areas identified as cytosol, nucleus, and mitochondria. A strong increase in the mitochondrial ratio occurred when the cytosolic ratio reached 4–5. These ratios are equivalent to a Ca$^{2+}$ activity of approximately 1200 nmol/liter in the cytosol.

**FIG. 10.** Relationship between the 345/380 nm ratio in the cytosol and the mitochondria after stimulation with carbachol (0.5–10 μmol/liter) (A) or thapsigargin (10–500 nmol/liter) (B). Separate linear correlations can be drawn in both figures for the two data groups from 0.5 to 4 and from 5 to 9. The switching point between the two data groups indicates the starting value for the fast [Ca$^{2+}$]$_{mic}$ increase. This again corresponds to a Ca$^{2+}$ activity of around 1200 nmol/liter in the cytosol.

**FIG. 11.** Original FDIM recording showing the effect of neurotensin (1 nmol/liter) on [Ca$^{2+}$]$_{cyt}$, [Ca$^{2+}$]$_{nuc}$, and [Ca$^{2+}$]$_{mic}$ in the absence of extracellular Ca$^{2+}$. The exclusive release of Ca$^{2+}$ from intracellular stores is sufficient to increase [Ca$^{2+}$]$_{cyt}$ in all areas, including [Ca$^{2+}$]$_{mic}$. The initial time course of [Ca$^{2+}$]$_{mic}$ is comparable to the control conditions. Similar results were obtained in four other experiments.
lowering the extracellular Ca\(^{2+}\) to 10\(^{-5}\) mol/liter after ionomycin application, which results in a drop of [Ca\(^{2+}\)]\(_{m}\) to or below the resting level of the cell (data not shown). From the in vitro fura-2 calibration curve, this is unexpected, as the fura-2 signal still should be nearly saturated at 10\(^{-5}\) mol/liter Ca\(^{2+}\).

However, taking into the account the described Ca\(^{2+}\) sensitivity of the capacitative Ca\(^{2+}\) entry mechanism in HT\(_{29}\) cells (22, 37), our data fit very well with the concept of an ionomycin-induced opening of the capacitative Ca\(^{2+}\) entry-influx pathway (35). Raising the ionomycin concentration to also enhance mitochondrial Ca\(^{2+}\) permeability resulted in a fast loss of the dye from the whole cell, including all cellular compartments. Other permeabilizing nonspecific agents, such as Triton and digitonin, gave similar results (data not shown; n = 5). Therefore, a direct permeabilization of mitochondria for Ca\(^{2+}\) was not possible in intact HT\(_{29}\) cells. Even the calibration of the cytosolic and the nuclear fura-2 signal with ionomycin has to be questioned in future studies, despite the fact that it is a well accepted and frequently employed method. From our spectroscopic measurements and the results of others (6, 8, 33), we propose that the fura-2 properties inside mitochondria are comparable to the cytosolic form. In addition, the results of our measurements could hardly be explained alone on the basis of changed fura-2 properties inside mitochondria. Due to the problems with an adequate calibration of [Ca\(^{2+}\)]\(_{m}\) we preferred to use, throughout this report, ratio values for [Ca\(^{2+}\)]\(_{m}\) except for some mean values, where we used the ionomycin calibration values from the cytosol despite the observed limitations of the procedure.

**Mitochondrial Ca\(^{2+}\) Response Is Dependent on Agonist Concentration**—A number of other laboratories have observed changes of [Ca\(^{2+}\)]\(_{m}\) in response to agonist stimulation in living cells using aequorin targeted to mitochondria (17, 18), fluo-3 (31), or dihydro-rhod-2 (17, 18). However, none of these studies reported the concentration response curve of [Ca\(^{2+}\)]\(_{m}\) and [Ca\(^{2+}\)]\(_{m}\) to agonists measured simultaneously and on the single cell level. This was due to limitations in sensitivity, limited optical resolution, and/or the dyes used.

With the higher concentrations of the agonists CCH, neuropeptide, and ATP (for ATP no data were shown), the response in [Ca\(^{2+}\)]\(_{m}\) to [Ca\(^{2+}\)]\(_{c}\), was biphasic. The initial rapid rise was only slightly slower than the cytosolic response, followed by a slower rise in [Ca\(^{2+}\)]\(_{m}\) when a threshold in [Ca\(^{2+}\)]\(_{c}\), was exceeded. Surprisingly, the response of [Ca\(^{2+}\)]\(_{m}\) to thapsigargin showed a similar [Ca\(^{2+}\)]\(_{c}\), dependence. The [Ca\(^{2+}\)]\(_{m}\), corresponding to the threshold value for agonists and thapsigargin, was estimated from the experiments shown in Fig. 10, A and B. A ratio of around 5 was obtained, corresponding to around 1200 nmol/liter. Around this cytosolic Ca\(^{2+}\) activity, a strong increase of [Ca\(^{2+}\)]\(_{m}\) could be observed. A similar, biphasic [Ca\(^{2+}\)]\(_{m}\) response has been described recently in endothelial cells after stimulation with bradykinin (31) and in cardiac myocytes after electrical stimulation (5).

Similar to the results of these studies, we could also not rule out the possibility that the initial, small and fast Ca\(^{2+}\) rise is the result of a spillover from the cytosolic signal due to a limited z resolution in conventional confocal microscopy and/or the movement of mitochondria. However, even when we used two-photon excitation of fura-2 (the method currently giving the highest possible z resolution for fast dynamic measurements) the results of the Ca\(^{2+}\) measurements were very similar. Therefore, it seems unlikely that the initial [Ca\(^{2+}\)]\(_{m}\) response is an artifact, even if FCCP did not influence it. In addition, in some of our experiments (for example, Figs. 9 and 11), we measured a discrepancy in the time course for this early phase of the [Ca\(^{2+}\)]\(_{m}\) and [Ca\(^{2+}\)]\(_{m}\), increase. This would indicate that, indeed, different compartments were contributing to the signal. It should also be pointed out that a
biphasic \([\text{Ca}^{2+}]_{\text{m}}\) rise was described in an isolated mitochondria preparation in response to \(\text{Ca}^{2+}\) pulses (38), which speaks against an optical artifact. The initial, fast \(\text{Ca}^{2+}\) increase could be caused by the large \(\text{Ca}^{2+}\) gradients postulated to exist at the beginning of a \(\text{Ca}^{2+}\) signal at sites of close contact to \(\text{Ca}^{2+}\)-release stores and mitochondria (39). In these so-called microdomains, very high \(\text{Ca}^{2+}\) activities of \(10^{-4}\) mol/liter were postulated.

All agonists could induce the biphasic increase of \(\text{Ca}^{2+}\) in mitochondria. The time course of the \(\text{Ca}^{2+}\) increase, as well as the observed differences between the cytosolic/nuclear and the mitochondrial concentration response curves (Figs. 3, 6, and 7), can be very well explained by the characteristics of the mitochondrial \(\text{Ca}^{2+}\) uptake mechanisms. The major uptake mechanism for \(\text{Ca}^{2+}\) in mitochondria is the \(\text{Ca}^{2+}\) uniporter (40), which is driven by the mitochondrial potential. Isolated mitochondria show a biphasic mode of \(\text{Ca}^{2+}\) uptake (38): the first rapid phase has a \(K_D\) of approximately 170 nmol/liter and inactivates within 1 s, and the second phase is activated at a \(\text{Ca}^{2+}\) starting rate > 400 nmol/liter. These values fit quite nicely with our results (Fig. 8, A and B) and with described relations of \(\text{Ca}^{2+}\) in cardiac myocytes after electrical stimulation (5).

**Physiological Role and Relevance of Changes of Mitochondrial \(\text{Ca}^{2+}\) Activity**—The measurement of cellular UV light-excited autofluorescence as a measurement of changes in the mitochondrial NADH levels in correlation with changes of \(\text{Ca}^{2+}\) has been used before by a number of groups, for example in myocytes (41), HeLa cells (39), and hepatocytes (17). The results of our measurement of autofluorescence (Fig. 10) are in good agreement with the measurements cited above. A number of mitochondria-specific enzymes, such as the three dehydrogenases for pyruvate, 2-oxoglutarate, and NAD\(^+\)-isocitrate (18, 42), are known to be stimulated by an increased \(\text{Ca}^{2+}\) in mitochondria. These enzymes increase ATP production when cytosolic ATP consumption is increased (40), and this helps the cell to keep pace with the higher energy demand.

In conclusion, we have shown, 1) using fura-2AM loading, that fura-2 accumulates in the mitochondria of HT\(_{29}\) cells in a \(\text{Ca}^{2+}\)-sensitive form; 2) that intramitochondrial fura-2 can be used to estimate the \(\text{Ca}^{2+}\) activity of mitochondria; 3) that inositol 1,4,5-trisphosphate-coupled agonists (CCH, neurotensin, and ATP) induce, in addition to their effect on \(\text{Ca}^{2+}\), and \(\text{Ca}^{2+}\) in a dose-dependent increase in the mitochondrial \(\text{Ca}^{2+}\) signal (the differentiated mitochondrial \(\text{Ca}^{2+}\) response may allow the cells to fine-tune their energetic response to hormone stimulation); and 4) that mitochondria in addition might serve as a sink or a modulator for \(\text{Ca}^{2+}\), and \(\text{Ca}^{2+}\).
Simultaneous Measurements of Cytosolic and Mitochondrial Ca\textsuperscript{2+} Transients in HT29 Cells

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