Calcium Dynamics in the Peroxisomal Lumen of Living Cells*‡§

Ilaria Drago†, Marta Giacomello‡, Paola Pizzo†, and Tullio Pozzan‡§

From the †Department of Biomedical Sciences and Consiglio Nazionale delle Ricerche Institute of Neuroscience, University of Padua and ‡Venetian Institute of Molecular Medicine, 35121 Padua, Italy

We here describe the generation of novel, green fluorescent protein-based Ca\(^{2+}\) indicators targeted to the peroxisome lumen. We show that (i) the Ca\(^{2+}\) concentration of peroxisomes in living cells at rest is similar to that of the cytosol; (ii) increases in cytosolic Ca\(^{2+}\) concentration (elicited by either Ca\(^{2+}\) mobilization from stores or Ca\(^{2+}\) influx through plasma membrane Ca\(^{2+}\) channels) are followed by a slow rise in intraperoxisomal [Ca\(^{2+}\)]; (iii) Ca\(^{2+}\) influx into peroxisomes is driven neither by an ATP-dependent pump nor by membrane potential nor by a H\(^+\)(Na\(^+\)) gradient. The peroxisomal membrane appears to play a low pass filter role, preventing the organelle from taking up shortlasting cytosolic Ca\(^{2+}\) transients but allowing equilibration of the peroxisomal luminal [Ca\(^{2+}\)] with that of the cytosol during prolonged Ca\(^{2+}\) increases. Thus, peroxisomes appear to be an additional cellular Ca\(^{2+}\) buffer, but their influx and efflux mechanisms are unlike those of any other cellular organelle.

A variation in cytosolic Ca\(^{2+}\) is a key component of the cell signaling machinery activated by receptor stimulation. Although a plethora of information is available regarding Ca\(^{2+}\) dynamics in different subcellular compartments, a notable exception is represented by peroxisomes, single membrane-bound organelles diffusely distributed within the cytosol of virtually all eukaryotic cells (1). Proteins located in the peroxisomal matrix are linked to different biochemical pathways (2) such as the \(\beta\)-oxidation of fatty acids and detoxification of hydrogen peroxide. The latter pathway is exclusively localized in the peroxisomal compartment of fungi and plants, whereas in mammalian cells it is distributed between peroxisomes and mitochondria (2). Specialized peroxisomal functions, such as fatty acid degradation and synthesis of phytohormones, are found in some cells, (e.g. plants and fungi) (3). Interest in peroxisomes has increased recently due to the discovery that defects in peroxisomal biogenesis and peroxisomal enzyme deficiencies are linked to several genetic disorders in humans (4). Given that any enzymatic activity is highly sensitive to the ionic composition of the surrounding environment, it is surprising that information on the luminal ion content of peroxisomes is scarce and contradictory. In particular, no data are currently available on Ca\(^{2+}\) concentration in the peroxisome lumen, [Ca\(^{2+}\)]\(_{p}\).

We here present a novel probe, derived from the new green fluorescent protein (GFP)\(^{2}\)-based Ca\(^{2+}\) indicators (Dcpv) (5), for monitoring [Ca\(^{2+}\)]\(_{p}\) in living cells. We show that peroxisomes contribute to the sequestration of part of the Ca\(^{2+}\) entering the cytoplasm during cell activation in a way that is unique among cellular organelles.

MATERIALS AND METHODS

Contrasts—The sequence coding for the tripeptide SKL was introduced before the stop codon of D3cpv (kindly provided by R. Tsien, San Diego, CA) by PCR using the oligonucleotides 5'-ACCCAAGCTTGGCCACCATG-3' (forward); 5'-ACCCAGCTTGGCCACCATG-3' (reverse). The resulting PCR product was digested with HindIII and EcoRI and ligated into pcDNA3 (Invitrogen). PCR for introducing the KVK coding sequence was performed using D3cpv-SKL as a template with the same forward primer and the following reverse: 5'-ACCCAAGCTTGGCCACCATG-3'. The cDNA of pHLucin was a kind gift from S. Grinstein (Toronto, Canada).

Cell Culture and Transfection—HeLa cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum supplemented with l-glutamine (2 mM), penicillin (100 units/ml), and streptomycin (100 \(\mu\)g/ml) in a humidified atmosphere containing 5% CO\(_2\), while GH3 cells were grown in the same medium supplemented with non-essential amino acid (Sigma). Cells were seeded onto glass coverslips (24-mm diameter); for GH3 cells, coverslips were pretreated with poly-L-lysine (50 \(\mu\)g/ml). Transfections were performed at 60% confluence using TransIT\(^{TM}\)-LT1 transfection reagent (Mirus, Bologna, Italy) with 1 \(\mu\)g of DNA. Fluorescence experiments were performed 48 h after transfection.

Cell Loading with Fura-2, BCECF, or BAPTA—To monitor cytosolic [Ca\(^{2+}\)] or pH, cells seeded on coverslips were incubated with 1 \(\mu\)M fura-2/AM or 2 \(\mu\)M BCECF/AM in an extracellular-like solution for 30 min at 37 °C, washed, and then incubated for 30 min at room temperature. In the experiments aimed at reducing cytosolic and organelle [Ca\(^{2+}\)] to the lowest possible level, cells were loaded contemporaneously with 1 \(\mu\)M fura-2/AM and 10 \(\mu\)M BAPTA/AM using the protocol described above in a medium without CaCl\(_2\) and supplemented with 500 \(\mu\)M EGTA.

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§ To whom correspondence should be addressed: Dept. of Biomedical Sciences, CNR Inst. of Neurosciences, University of Padua, Viale G Colombo 3, 35121 Padua, Italy. Tel: 39-049-827-6067; Fax: 39-049-827-6049; E-mail: tullio.pozzan@unipd.it.

1 The abbreviations used are: GFP, green fluorescent protein; TRH, thyrotropin-releasing hormone; BCECF, 2,7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein; BAPTA, 1,2-bis(o-aminophenoxy)ethane-N,N',N",N"-tetraacetic acid.

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Cell Imaging—Cells expressing (or loaded with) the fluorescent probes were analyzed using an inverted fluorescence microscope (Zeiss Axioplan) with an immersion oil objective (×63, N.A. 1.40, for fluorescent probes and ×40, N.A. 1.3, for foru-2 and BCECF). Excitation light was produced by a monochromator (Polychrome II; TILL Photonics, Martinsried, Germany): 400 and 480 nm for pHluorin; 340 and 380 nm for foru-2; 495 and 440 nm for BCECF. The two excitation wavelengths were rapidly alternated and the emitted light deflected by dichroic mirrors (HQ 520 LP for pHluorin and BCECF and 455 DRPL for foru-2) was collected through emission filters (HQ 520 LP for pHluorin and BCECF and 480 ELFP for foru-2). For the D3-derived probe, the excitation light was 425 nm. The emitted light was collected through a beam splitter (OES s.r.l., Padua, Italy) (emission filters HQ 480/40M for cyan fluorescent protein and HQ 535/30 M for yellow fluorescent protein) and a dichroic mirror (515 DCXR). Filters and dichroic mirrors were purchased from Omega Optical and Chroma. Images were acquired using a cooled CCD camera (Imago; TILL Photonics) attached to a 12-bit frame grabber. Synchronization of the monochromator and CCD camera was performed through a control unit run by TILLvisION v.4.0 (TILL Photonics); this software was also used for image analysis. For time course experiments, the fluorescence intensity was determined over regions of interests covering small groups of peroxisomes or cytosolic regions (devoid of identifiable structures). Exposure time and frequency of image capture varied from 30 to 500 ms and from 5 to 0.2 Hz, respectively. Cells were mounted into an open-topped chamber thermostated at 37 °C and maintained in an extracellular medium containing (in mM): 135 NaCl, 5 KCl, 1 MgSO4, 0.4 KH2PO4, 10 glucose, 20 Hepes, pH 7.4, at 37 °C. Plasma membrane permeabilization was performed by treating cells for 1 min with 100 μM digitonin (which results in complete release of the cytosolic probe), punctate structures scattered throughout the cytoplasm, later verified to be peroxisomes (see “Results” for details), became visible (C).

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**FIGURE 1. Subcellular distribution of transiently expressed D3cpv-SKL in HeLa cells.** D3cpv, a new generation member of the Cameleon, fluorescence resonance energy transfer-based Ca2+ sensor family, carrying new mutations (*) in the Calmodulin (CaM) and M13 sequences (5), was modified by insertion at the C terminus of the peroxisome targeting signal SKL (upper panel). HeLa cells transiently expressing the new protein (A) or the original cytosolic probe (B) showed no difference in their subcellular distribution. Upon plasma membrane permeabilization with 100 μM digitonin, evidence for partial release of the cytosolic probe (C) is visible (C).

RESULTS

**Peroxisome Targeting of the GFP-based Ca2+ Indicator**—Fig. 1, top, shows the schematic structure of D3cpv, modified by the insertion at the C-terminal of the canonical peroxisomal targeting signal, the tripeptide Ser-Lys-Leu (SKL) (6). Although this sequence is known to be efficacious in targeting several recombinant proteins to peroxisomes, the D3cpv-SKL subcellular distribution (Fig. 1A) in HeLa cells transiently expressing the construct was indistinguishable from that of cytosolic D3cpv (Fig. 1B). Treatment of cells with digitonin, although releasing all cytosolic D3cpv (not shown), revealed that a fraction of the D3cpv-SKL was trapped in numerous small structures scattered throughout the cytoplasm (Fig. 1C). The D3cpv-SKL-positive spots coincide with peroxisomes, as revealed by their positivity after immunostaining with antibodies for markers of these organelles, catalase (Fig. 2B) or the peroxisomal membrane protein 70 (Fig. 2E). The misorting of D3cpv-SKL was observed in all other cell types investigated, GH3, Chinese hamster ovary, and SH-SY5Y. The cytoplasmic staining was not due to protein overexpression and saturation of the peroxisome protein import mechanism, because the same results were obtained when transfection was carried out with only 1/5 of the cDNA or if the cells were observed 48, 72, or 96 h after transfection (not shown).
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To improve the peroxisome localization, a novel construct was made where the C-terminal SKL was preceded by a three-amino acid positively charged sequence, Lys-Val-Lys (KVK). This sequence was designed to fit the requirement for improved peroxisomal targeting described by Neuberger et al. (7, 8) (Fig. 3A). The majority of cells transfected with D3cpv-KVK-SKL were characterized by the presence only of punctate fluorescence, with a negligible signal in the cytosol. A small percentage of cells (5–30%) still revealed cytosol missorted probe. Such increase in targeting efficiency was observed also in Chinese hamster ovary, SH-SY5Y (not shown), and GH3 cells (Fig. 3D). Immunostaining with anti-PMP70 antibody revealed that all the D3cpv-KVK-SKL-positive vesicles of HeLa and GH3 cells are also positive for the bona fide peroxisome marker (Fig. 3, C and F).

The experiment presented in Fig. 3G was aimed at determining whether the Ca\(^{2+}\) probe was trapped within the peroxisome lumen or whether it was bound to the cytosolic surface of peroxisomes. The plasma membrane of HeLa cells was permeabilized with digitonin, and the cells were then treated with Proteinase K. The protease did not affect the D3cpv-KVK-SKL fluorescent signal, whereas, on the contrary, in cells expressing a GFP construct localized on the cytosolic surface of the outer mitochondrial membrane, TOM20-GFP (9), the enzyme abolished the fluorescence in a few seconds. Similar results were obtained in GH3 cells (not shown).

Ca\(^{2+}\) Handling by Peroxisomes in Intact Cells—We used as a first model system GH3 cells because these cells are endowed with (i) abundant plasma membrane voltage-gated Ca\(^{2+}\) channels and (ii) endogenous receptors (TRH receptors) coupled to inositol 1,4,5-trisphosphate production and Ca\(^{2+}\) mobilization from stores (10). Fig. 4A shows the typical response pattern of the D3cpv-KVK-SKL fluorescence signal of three GH3 cells to depolarization with 30 mM KCl. In two cells, the fluorescence was exclusively in peroxisomes, whereas in the third cell fluorescence was diffuse throughout the whole cytosol. Cell depolarization caused an increase in the fluorescence emitted at 540 nm and a decrease of the signal at 480 nm (not shown) and thus an increase in the 540/480-nm fluorescence emission ratio (here presented as \(\Delta R/R_0\), which is proportional to \([\text{Ca}^{2+}]\) (Fig. 4A). The kinetics of the \(\Delta R/R_0\) changes were, however, different in the cells where the probe was localized in the peroxisomes and in the cell with the mistargeted indicator. The cytosolic \(\Delta R/R_0\) (continuous line) reached the peak in 1–2 s and then started to decrease slowly; the peroxisome signal, on the contrary, reached the peak in 10–15 s and then started to decline. Addition of EGTA accelerated the drop to basal level of both the cytosolic and peroxisomal signals, the effect on the cytosol being more evident. In Fig. 4B, the fluorescence emission ratio (excitation 340/380 nm) of a parallel batch of cells loaded with the Ca\(^{2+}\) indicator fura-2 is presented. The kinetics of the fura-2 signal were similar to that of cells expressing the missorted D3cpv-KVK-SKL probe (Fig. 4A). Similar data were obtained with cells expressing the original cytosolic D3cpv (not shown). In the experiment presented in Fig. 4C the peak levels reached by \([\text{Ca}^{2+}]\) expressed as percentage of the maximal
ΔR/Ro are plotted as a function of KCl concentration. For comparison, in Fig. 4D the 340/380 nm emission ratio of cells loaded with fura-2 is also shown. It is clear that the peak rise in peroxisomal Ca2+ was observed (not shown).

The question then arises as to the behavior of peroxisomes, in terms of Ca2+ response, to agents that cause Ca2+ mobilization from intracellular stores, either elicited by TRH or by the Ca2+ ionophore ionomycin, both added in the absence of extracellular Ca2+. The two agents caused either a drop or a rise in [Ca2+]p (under conditions that elicited significant transient Ca2+ rises, as measured with fura-2; compare Fig. 5, A and C, with Fig. 5, B and D, dotted traces). When TRH or ionomycin was added after a previous pulse of KCl (to overload Ca2+ stores), the percentage of peroxisomal Ca2+ increases in response to TRH and ionomycin increased significantly (20 and 53% of cells, respectively; not shown). The problem of the peroxisomal behavior in response to Ca2+-mobilizing stimuli was then further addressed in HeLa cells treated with histamine or ionomycin (Fig. 5). In all cells investigated, histamine induced a cytosolic [Ca2+] rise, as measured with fura-2 (Fig. 5B, continuous trace), whereas in 68% of cells the peroxisome signal also increased significantly (Fig. 5A, continuous trace). In HeLa cells, addition of ionomycin in Ca2+-free medium (which resulted in a large cytosolic [Ca2+] increase in all cells tested; Fig. 5D, continuous trace) always resulted in a rise of [Ca2+]p. As shown in Fig. 6A, Ca2+ uptake was similar with (continuous trace) and without (dotted trace) an energy source. Notably, when an excess EGTA was added (to rapidly decrease medium [Ca2+]), the peroxisome [Ca2+] decreased with relatively slow kinetics (Fig. 6B). To test whether peroxisomal Ca2+ influx depends on the presence of a classical Ca2+ channel, digitonin-permeabilized cells were treated with 10 μM La3+, a nonspecific inhibitor of several Ca2+ channels. The increase in [Ca2+]p upon increase in medium [Ca2+] to 500 nM or 5 μM was unaffected by La3+ (not shown).

We then investigated whether peroxisomal Ca2+ uptake may depend on a Na+/Ca2+ antiport. Intact GH3 cells were pretreated with either NH4Cl (Fig. 6C, dotted trace), an agent that causes an alkalization of organelle pH, or monensin (dashed trace), a H+/Na+ exchange ionophore, which should collapse any gradient of either Na+ or H+ across the peroxisomal membrane, if they exist. Neither NH4Cl nor monensin had any appreciable effect on the [Ca2+]p increase caused by 30 mM KCl. Similar results were obtained in HeLa cells stimulated with either histamine or ionomycin (not shown).
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To verify whether there are heterogeneities among the organelles, the [Ca\(^{2+}\)] rise in different groups of peroxisomes was next investigated. As shown in Fig. 6D, the response to a 30-mM KCl challenge of different groups of organelles within the same GH3 cell was found to be very similar. Identical results were obtained in HeLa cells using either ionomycin or histamine as the stimulus (not shown).

Finally, the peroxisome lumenal pH was directly monitored using the targeted pH indicator pHluorin (see “Materials and Methods” and Ref. 11). Cytosolic pH was measured in parallel with BCECF (12). Fig. 7 shows that the weak acid acetate caused a reduction of both cytoplasmic (Fig. 7A) and peroxisomal (Fig. 7B) pH, whereas NH\(_4\)Cl caused an alkalinization of both compartments. Monensin also caused an increase of pH both in the cytosol (Fig. 7C, continuous trace) and in peroxisomes (Fig. 7D, dotted trace). When the cells were incubated in a medium where NaCl was iso-osmotically substituted with KCl (to abolish the Na\(^+\) gradient across the plasma membrane and in the absence of Ca\(^{2+}\) to block Ca\(^{2+}\) influx) and the extracellular pH was dropped to 7.0 (to reduce the pH gradient), monensin hardly modified cytosolic pH (Fig. 7C, dashed point trace) and in parallel failed to cause any significant change in peroxisomal pH (Fig. 7D, dashed trace).

Calibration of the Peroxisomal [Ca\(^{2+}\)]—To determine the absolute values of [Ca\(^{2+}\)]\(_p\), the in situ K\(_d\) for Ca\(^{2+}\) of D3cpv-KVK-SKL was determined using the passive Ca\(^{2+}\) loading procedure previously described (13). Transfected cells were permeabilized with digitonin in an intracellular-like medium, but devoid of ATP or any mitochondrial oxidizable substrate, and variable concentration of Ca\(^{2+}\) (see “Materials and Methods”). The percentage of the normalized 540/480-nm fluorescence emission ratio changes at steady state were then plotted as a function of medium [Ca\(^{2+}\)] (Fig. 8). The apparent K\(_d\) for Ca\(^{2+}\), as calculated in situ, 1.0 \(\mu\)M, is not much different from that calculated in vitro with recombinant D3cpv, 0.6 \(\mu\)M (5). A detailed description of the protocol employed to calculate the [Ca\(^{2+}\)] within peroxisomes is presented in supplemental data. A summary of the absolute values of [Ca\(^{2+}\)]\(_p\) (as measured with D3cpv-KVK-SKL), compared with the cytosolic Ca\(^{2+}\) values (as
measured in parallel by fura-2), is presented in Table 1. The $[\text{Ca}^{2+}]_{\text{p}}$ at rest, both in HeLa and GH3 cells, is $\sim 150 \text{ nM}$, i.e. not significantly different from that measured with fura-2 (150 and 190 $\text{ nM}$ for GH3 and HeLa cells, respectively). The mean $[\text{Ca}^{2+}]_{\text{p}}$ peak of GH3 cells (upon stimulation with 30 $\text{ mM}$ KCl), $1.32 \pm 0.25 \text{ $\mu$M}$, compares to $3.34 \pm 0.84 \text{ $\mu$M}$ measured with fura-2. The averaged $[\text{Ca}^{2+}]_{\text{p}}$ peaks in HeLa cells stimulated with histamine or ionomycin (both added in $\text{Ca}^{2+}$-free medium) are $0.72 \pm 0.11$ and $0.76 \pm 0.11 \text{ $\mu$M}$ compared with cytosolic peaks of $1.31 \pm 0.13$ and $1.33 \pm 0.59 \text{ $\mu$M}$, respectively.

**DISCUSSION**

The most common peroxisome-targeting mechanism involves the C terminus tripeptide SKL (6). When this sequence was added to the GFP-based $\text{Ca}^{2+}$ indicators D1- and D3cpv (5) most of the transfected protein mislocalized to the cytosol. Inclusion of a longer targeting sequence (KVK-SKL), however, resulted in more satisfactory peroxisome localization. The expressed protein is clearly trapped in the lumen of the organelles, as demonstrated by its resistance to proteolytic cleavage and by the slower kinetics of the fluorescence signal changes in response to a sudden change in extraperoxisomal $[\text{Ca}^{2+}]_{\text{p}}$.

When cytosolic $[\text{Ca}^{2+}]_{\text{p}}$ was increased in GH3 cells by depolarizing the plasma membrane with high KCl, the $[\text{Ca}^{2+}]_{\text{p}}$ also raised, although with slower kinetics. The amplitude of the $[\text{Ca}^{2+}]_{\text{p}}$ increase paralleled that of the cytosol. In quantitative terms, the maximum rises of $[\text{Ca}^{2+}]_{\text{p}}$ after depolarization were lower than those calculated with the classical cytosolic indicator fura-2. Considering the inherent assumptions involved in the calibration procedures of the two probes, it can be safely concluded that $[\text{Ca}^{2+}]_{\text{p}}$ tends to equilibrate with the cytosolic $[\text{Ca}^{2+}]$ and no driving force (ATP and/or $\text{Na}^+$ ($\text{H}^+$) gradients) leads to $\text{Ca}^{2+}$ influx into peroxisomes. In support of this conclusion, the luminal pH of peroxisomes is practically indistinguishable from that of the cytoplasm, and monensin never caused an acidification of peroxisomal lumen, demonstrating that $[\text{Na}^+]$ of peroxisomes is similar to that of cytoplasm. Our conclusion concerning the lack of any significant gradient of $\text{H}^+$ across the peroxisomal membrane concurs with Jankowski et al. (11), whereas other groups have reported that the intraperoxisome pH is slightly alkaline in mammalian cells (14) or in yeasts slightly acidic (15) or alkaline (16, 17).

A permeability barrier to $\text{Ca}^{2+}$ diffusion across the peroxisome membrane, however, does exist as demonstrated by these results: (i) the rate of peroxisome $\text{Ca}^{2+}$ rise in intact cells treated with KCl is substantially slower than in the cytosol, and (ii) in permeabilized cells, sudden changes in medium $[\text{Ca}^{2+}]$ require several seconds to equilibrate with the organelle lumen. Surprisingly, whereas increases in cytosolic $[\text{Ca}^{2+}]_{\text{p}}$ elicited in GH3 cells by $\text{Ca}^{2+}$ influx though voltage-gated $\text{Ca}^{2+}$ channels were followed by $[\text{Ca}^{2+}]_{\text{p}}$ rises, $\text{Ca}^{2+}$ mobilization from internal stores, as induced by stimulation of TRH receptors, almost never resulted in a significant increase in $[\text{Ca}^{2+}]_{\text{p}}$. Even specific $\text{Ca}^{2+}$ mobilization from stores, as promoted by ionomycin added in $\text{Ca}^{2+}$-free medium, was unable to induce $\text{Ca}^{2+}$ uptake into peroxisomes of GH3 cells. The possibility was thus considered that the poor response of the peroxisomes to $\text{Ca}^{2+}$ mobilization in GH3 cells reflects (i) the existence of a mechanism that prevents $\text{Ca}^{2+}$ uptake in peroxisomes in response to $\text{Ca}^{2+}$ mobilization from stores or (ii) a combination of the small and transient nature of the cytosolic $\text{Ca}^{2+}$ rise in response to TRH (and ionomycin) in GH3 cells and of the slow $\text{Ca}^{2+}$ uptake rate by peroxisomes. In other words, the small and transient rise in cytosolic $[\text{Ca}^{2+}]$ (as that elicited in GH3 cells by TRH or ionomycin) can be hardly coped with by the relatively slow $\text{Ca}^{2+}$ uptake system of peroxisomes. The cytoplasmic $[\text{Ca}^{2+}]$ rise in response to depolarization, instead, does reach the peak in 2–3 s, but it is followed by a prolonged plateau level that lasts several tens of seconds. In support of the latter explanation, the very rapid $\text{Ca}^{2+}$ increases due to spontaneous action potential firing (and $\text{Ca}^{2+}$ influx through voltage-gated $\text{Ca}^{2+}$ channels) often observed in GH3 cells (18) were never followed by significant increases in $[\text{Ca}^{2+}]_{\text{p}}$.

To distinguish between these possibilities, we used a different cell type, HeLa, where $\text{Ca}^{2+}$ mobilization from stores in response to an inositol 1,4,5-trisphosphate-generating agonist, such as histamine, results in larger and relatively more prolonged $\text{Ca}^{2+}$ transients compared with GH3 cells (peak values measured with fura-2 of 1.31 $\mu$M and 270 $\text{ nM}$, back to basal levels in 120 and 50 s, in HeLa and GH3 cells, respectively). Indeed, we found that in HeLa cells the percentage of peroxisome responses to histamine application was much higher than that observed in GH3 cells in response to TRH (68 versus 1%, respectively) and the percentage of $[\text{Ca}^{2+}]_{\text{p}}$ increases in response to ionomycin was close to 100% in HeLa cells compared with <5% in GH3 cells. Thus, it may be concluded that, due to the intrinsic sluggish response to a cytosolic $\text{Ca}^{2+}$ rise, peroxisomes are relatively insensitive to rapid transients of cytosolic $[\text{Ca}^{2+}]$ but significantly increase their $\text{Ca}^{2+}$ level only in response to prolonged cellular $\text{Ca}^{2+}$ increases. We cannot exclude, however, that peroxisomes of HeLa cells are more efficient than those of GH3 cells at taking up $\text{Ca}^{2+}$. However, when in GH3 cells TRH- or ionomycin-induced cytosolic $\text{Ca}^{2+}$ increases are larger and more prolonged (as occurs when they are applied after KCl), the percentage of peroxisomal responses increases drastically (from 1 to 21% for TRH and from 5 to 53% with ionomycin), suggesting that the first explanation is most likely.

The final question concerns the heterogeneity of peroxisomal $\text{Ca}^{2+}$ responses. When groups of organelles in the same cell were compared, no significant difference, either in kinetics or in amplitude of the $\text{Ca}^{2+}$ responses, was ever observed. It cannot

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**TABLE 1**

| Stimulus          | Cell type | $\text{Ca}^{2+}$ | S.E. | $\text{Ca}^{2+}$ | S.E. |
|------------------|-----------|-----------------|-----|-----------------|-----|
|                  |           | fura-2          |     | D3cpv-KVK-SKL   |     |
| $[\text{KCl}]$  | GH3       | 1.79            | 0.47| 0.52            | 0.08|
| ($10 \text{ mM}$) |           | 2.01            | 0.41| 0.64            | 0.10|
| ($10 \text{ mM}$) | GH3       | 3.34            | 0.84| 1.32            | 0.25|
| Histamine        | HeLa      | 1.31            | 0.13| 0.72            | 0.11|
| ($100 \text{ nM}$) |           | 1.33            | 0.59| 0.76            | 0.11|

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be excluded, however, that single organelles localized in the proximity of Ca\(^{2+}\) channels of either the plasma membrane or the endoplasmic reticulum may experience larger local Ca\(^{2+}\) rises and, accordingly, undergo larger Ca\(^{2+}\) increases.

In conclusion, we have developed novel GFP-based Ca\(^{2+}\) indicators that can efficiently target to the peroxisomal lumen. These allow, for the first time to our knowledge, the measurement of this parameter in intact living cells. Taken together, the present data demonstrate that peroxisomes participate in the microbial cycles of Ca\(^{2+}\) signaling pathway but their behavior is unlike that of any other organelle. In particular, peroxisomes do not act as Ca\(^{2+}\) stores from which Ca\(^{2+}\) can be mobilized upon stimulation, as the endoplasmic reticulum, the Golgi apparatus or, in some cells, acidic compartments (19). The Ca\(^{2+}\) response of peroxisomes to a rise in cytosolic [Ca\(^{2+}\)]\(_c\) is also markedly different from that of mitochondria, in as much as their luminal Ca\(^{2+}\) does not increase as massively as that of the latter organelles.

The organelle that most resembles peroxisomes in terms of Ca\(^{2+}\) response is the nucleus, although in the latter the kinetics of Ca\(^{2+}\) equilibration with the cytosol are 10–100-fold faster. Thus, because of this relatively slow Ca\(^{2+}\) influx, very rapid and transient increases in cytosolic Ca\(^{2+}\) may not lead to appreciable changes in [Ca\(^{2+}\)]\(_p\), whereas more sustained increases will always lead to an increase in [Ca\(^{2+}\)]\(_p\). It remains to be established whether and which reactions within the peroxisomes are affected by Ca\(^{2+}\).

The amount of Ca\(^{2+}\) that is sequestered by peroxisomes will depend on (i) their number and volume (which may vary among different cells and in response to specific stimuli, e.g. peroxisome proliferator-activated receptor \(\gamma\) gene activation) and (ii) the endogenous Ca\(^{2+}\) buffering capacity of the organelles, which is presently unknown. In addition to a potential role as a cytosolic Ca\(^{2+}\) buffer, the increases in [Ca\(^{2+}\)]\(_p\) may be relevant for the organelle’s own functions. Thus far, potential candidates are the peroxisomal Ca\(^{2+}\)–dependent members of the mitochondrial carrier superfamily that contains four EF-hand Ca\(^{2+}\) binding domains (20) or a Ca\(^{2+}\)/calmodulin-regulated catalase isoform found in plant peroxisomes (21). The search for Ca\(^{2+}\)-modulated peroxisomal proteins may now be launched on a firmer ground, given the direct demonstration of the participation of these organelles in cellular Ca\(^{2+}\) handling.

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