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BCECF IN SINGLE CULTURED CELLS: INHOMOGENEOUS DISTRIBUTION BUT HOMOGENEOUS RESPONSE

MICHAEL WEINLICH*, CHRISTIANE THEIß, CHIANN-TSO LIN AND ROLF K. H. KINNE
Max-Planck-Institut für molekulare Physiologie, Postfach 10 26 64, 44026 Dortmund, Germany
*Present address: Chirurgische Universitätsklinik Tübingen, Hoppe-Seyler-Straße3, 72076 Tübingen, Germany
(e-mail: michael.weinlich@uni-tuebingen.de)

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Summary

Using confocal laser scanning microscopy with a dual-wavelength laser system, the behaviour of BCECF [(2′,7′-bis-2-carboxyethyl)-5-(and-6)carboxyfluorescein] was investigated in a variety of cell lines. Selection of a small area for monitoring allowed discrimination between various intracellular organelles, whose identity was established by vital staining. It was found that, after loading the cells with BCECF, both the nucleus and the mitochondria showed a higher level of fluorescence than the cytoplasm. Calibration of the pH-sensitivity of these fluorescence signals using the nigericin method yielded identical curves, as did exposure of the cells to NH_4Cl. These studies suggest that BCECF, despite its inhomogeneous intracellular distribution, reports the pH of only one cellular compartment, the cytosol.

Key words: confocal laser scanning microscopy, (2′,7′-bis-2-carboxyethyl)-5-(and-6)carboxyfluorescein, hydrogen ion concentration, mitochondria, cellular compartments, nucleus, cytoplasmic pH.

Introduction

BCECF has proved to be a reliable indicator of intracellular pH (Tsien, 1989). In most cases, intracellular pH is determined by collecting the fluorescence signal from several cells. Since cells are compartmentalised and the pH of different compartments such as endosomes, lysosomes and mitochondria differs from that of the cytoplasm, it is necessary to know the extent to which the various compartments contribute to measurements of intracellular pH.

Several authors have reported an uneven distribution of BCECF within cells (Bright *et al.* 1989; Rosenberg *et al.* 1991; Slayman and Moussatos, 1994; Wang and Kurtz, 1990) and although Paradiso *et al.* (1986) revealed an even distribution of BCECF within gastric glands, they demonstrated that BCECF might not be localised to mitochondria, because no pH changes were seen when the cells were exposed to valinomycin or rotenone. The relevance of uneven dye distribution for accurate intracellular pH measurements is, therefore, still a matter for discussion (Slayman and Moussatos, 1994).

In the current study, this problem was investigated using dual-wavelength confocal laser scanning microscopy allowing both the investigation of small intracellular compartments by optical sectioning and pH measurements using ratiometry.

Materials and methods

Chemicals and solutions

Stock solutions of BCECF-AM ester (10 mmol l^{-1} in dimethyl sulphoxide) and nigericin [10 mmol l^{-1} in dimethylformamide (DMF)/ethanol 3:1], both from Molecular Probes, Eugene, OR, USA, were stored at –20 °C. All other chemicals were obtained from Sigma (St Louis, MO, USA). The perfusion solution (modified Ringer's solution) contained (in mmol l^{-1}): 128 NaCl, 5.4 KCl, 0.3 Na_2HPO_4, 1.8 $MgSO_4$, 1 $CaCl_2$, 1 glucose, 14 L-alanine and 25 Hepes. Using *N*-methyl-D-glucamine (NMG), pH was adjusted to 7.4 at 37 °C. In NH_4Cl-containing solutions, 20 mmol l^{-1} Na^+ was replaced by 20 mmol l^{-1} NH_4^+. The calibration solution with high [K^+] contained (in mmol l^{-1}): 140 KCl, 1.8 $MgSO_4$, 1 $CaCl_2$, 1 glucose, 10 Hepes, 14 L-alanine and 10 µmol l^{-1} nigericin. pH was adjusted to 6.5, 7.2 and 7.9 at 37 °C.

Cell lines

TALH SVE.1 cells are an immortalized cell line from the medullary thick ascending limb of Henle's loop (TALH) of rabbit kidney (Scott *et al.* 1986; von Recklinghausen *et al.* 1992). E49 cells are an endothelial cell line. The LLC-PK_1 cell line, a renal epithelial cell line (Jans *et al.* 1987) with characteristics of proximal tubular cells, was obtained from the laboratory of Professor Dr Heini Murer (Zürich, Switzerland). TALH cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium), E49 cells were maintained in Ham's F-12 medium and LLC-PK_1 cells were cultured in Eagle's minimal essential medium. All of the above media were supplemented with non-essential amino acids, glutamine and 10 % foetal calf serum. Cell lines were maintained in stock culture at a subconfluent density in 75 cm^2 tissue-culture flasks in a humidified 5 % CO_2 atmosphere at 37 °C. Cells were detached from the culture dishes with 0.5 g l^{-1} trypsin, 0.2 g l^{-1} EDTA in Duck's buffer. Cells were then allowed to grow on coverslips and used for experiments before they became confluent.

Staining of cells

Vital stains were used in all experiments. Cells were loaded with 10 μmol l^{-1} BCECF-AM at 37 °C for 15 min. After rinsing, cells were allowed to recover for a post-loading period of 30 min. Mitochondria were stained with a 10 μg ml^{-1} rhodamine 123 solution for 10 min at room temperature (21 °C) in Ringer's solution (Johnson *et al.* 1980). Endoplasmic reticulum was stained with $DiOC_6$ (3,3′-dihexyloxacarbocyanine iodide) using a stock solution of 0.5 mg ml^{-1} $DiOC_6$ in ethanol diluted to 0.5 μg ml^{-1} in Ringer's solution. Cells were stained for 10 min at room temperature (Terasaki *et al.* 1984). To stain the Golgi apparatus, a stock solution of 0.576 mg ml^{-1} C_6NBD {N-[7–4(nitrobenz-2-oxa-1,3-diazol)]-6-aminocaproyl sphingosine}-ceramide in DMSO was used. Cells were exposed to 10 nmol l^{-1} C_6NBD-ceramide in Ringer's solution for 10 min at 37 °C (Lipsky and Pagano, 1985).

After dye-loading, the coverslips with the cells were mounted upside-down in a microperfusion chamber. Cells were superfused with Ringer's solution provided by a hydrostatic perfusion system.

Confocal laser scanning microscopy

Cells were examined using a modified MRC-600 confocal laser scanning system (BioRad, Hemel Hempstead, UK) connected to a Nikon Labophot epifluorescence microscope. To examine the behaviour of BCECF in dual-excitation mode, two laser sources were mounted to the confocal laser scanning system as described previously (Opitz *et al.* 1991). The excitation light at 488 nm (pH-dependent wavelength) was provided by an argon ion laser (Ion Laser Technology, Salt Lake City, UT, USA). A He–Cd laser (4130 N; Liconix, Santa Clara, CA, USA) was used to adapt the confocal laser scanning microscope for excitation at 442 nm (pH-independent wavelength) (Wang and Kurtz, 1990; Weinlich *et al.* 1994). To reduce photobleaching, a 1 % neutral density filter was introduced into the light paths, and the laser beams were scanned across the specimen only once for each image. One scan for a 512×768 pixel image lasted approximately 1 s. The light emitted above 515 nm was collected by a photomultiplier. A pair of digitised images, collected within 2 s, was obtained every minute and stored on hard disk.

Certain criteria were applied to distinguish between different compartments. In the confocal fluorescence image, the nucleus could easily be identified by its size and round shape. The compartments of interest that stained after BCECF loading were considered to be outside the nucleus and had a stronger fluorescence intensity than their surroundings. The cytoplasm was defined as the homogeneous space outside the nucleus and between the compartments of interest.

Cells were examined, using a ×60 oil-immersion objective (Nikon, Düsseldorf, Germany) with a numerical aperture of 1.40. The experimental determination of the depth of field of the optical arrangement (Weinlich *et al.* 1993) was performed by optically sectioning small fluorescent beads (0.1 μm, excitation at 442 nm; Molecular Probes, Eugene, OR, USA) at consecutive focus levels. The measured depth of field was 0.32 μm.

Measurements of pH

Intracellular pH measurements from cells stained with BCECF were carried out after the cells had reached a steady state in the perfused microchamber. Resting pH measurements were performed on TALH, LLC-PK_1 and E49 cells. Four ratio images with excitations at 442 nm and at 488 nm were obtained every minute followed immediately by nigericin calibration with buffers of pH 6.5, 7.2 and 7.9. Absolute intracellular pH was calculated by using the fluorescence intensity of a small area of interest inside the compartments of the cells. Such areas contained approximately 50–100 pixels each.

Changes in intracellular pH were produced using the NH_4Cl prepulse technique (Roos and Boron, 1981). After 4 min of steady-state pH measurement, cells were exposed to 20 mmol l^{-1} NH_4Cl buffer for 4 min. NH_4Cl buffer was then replaced with plain NaCl buffer for another 4 min. In cyanide experiments, 5 mmol l^{-1} KCN was used for 10 min. Each experiment was immediately followed by nigericin calibration. The same area of interest within the cell was observed throughout the whole experiment and the calibration procedure.

Calculation of the absolute intracellular pH was assessed using ratiometry and nigericin calibration (Thomas *et al.* 1982). The mean fluorescence intensity of the area of interest obtained at 488 nm was divided by that at 442 nm, yielding the ratio. Autofluorescence at both wavelengths was approximately ten times lower than BCECF fluorescence and was automatically subtracted from BCECF fluorescence by adapting the lowest fluorescence intensity on the grey scale from 0 to 256 just above the level of autofluorescence. A linear calibration curve was calculated using the ratios obtained at the three calibration steps. This curve, which was calculated for each experiment and for each compartment, was used to calibrate the absolute intracellular pH of each compartment. By using ratiometry, the calculated pH values were independent of dye concentration or volume changes, even within compartments.

Statistical analysis

Results are expressed as means ± S.E.M. Statistical significance was evaluated by paired or unpaired Student's *t*-test. $P<0.05$ was considered significant.

Results

Distribution of BCECF fluorescence

The fluorescence in BCECF-stained cells (Fig. 1, first row) was not homogeneously distributed in any of the three cell lines investigated. As expressed quantitatively in Table 1, three major

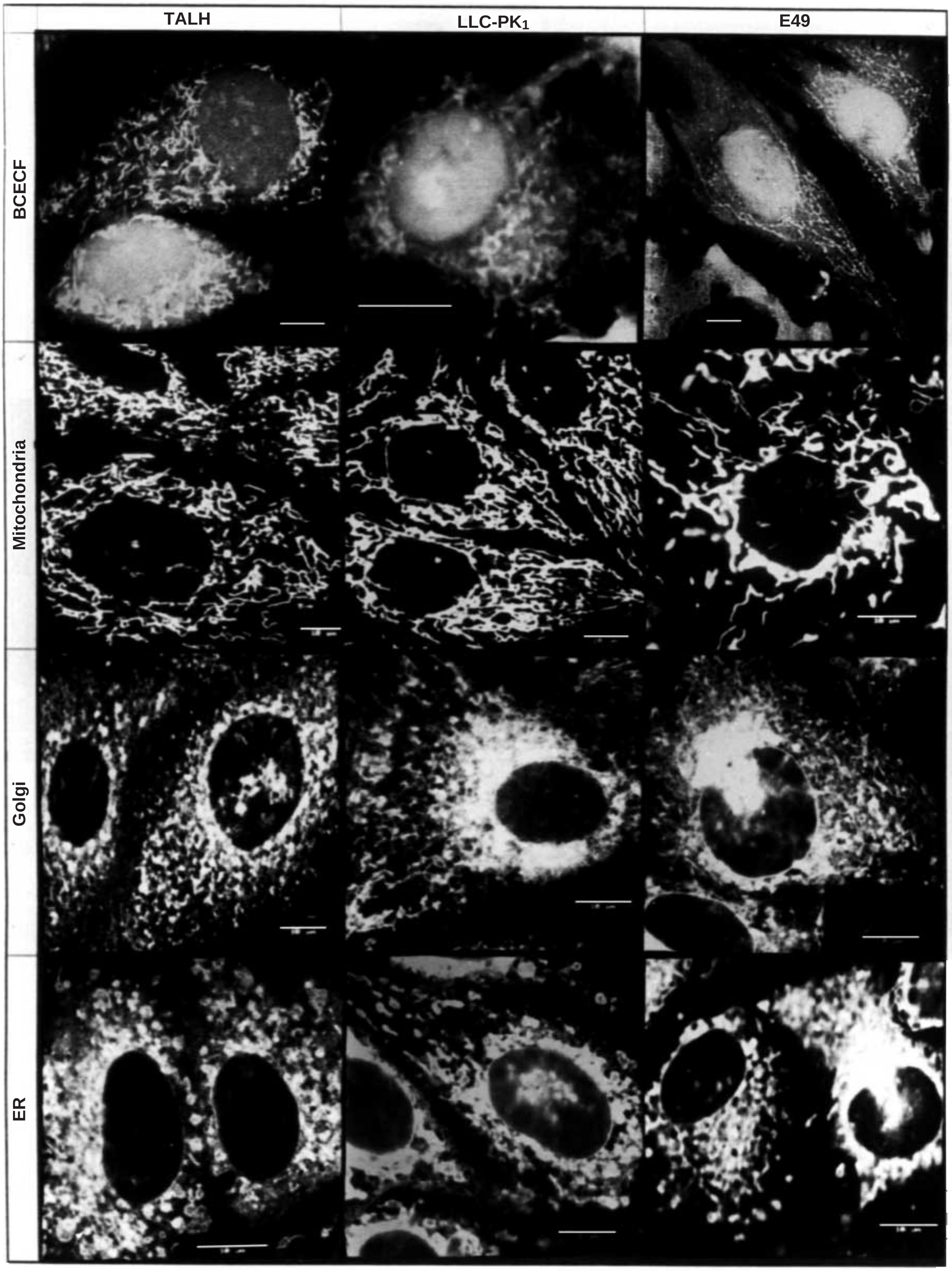

Fig. 1. Confocal laser scanning image of TALH, LLC-PK_1 and E49 cells stained with BCECF or vital dyes to visualise intracellular compartments. Cells were loaded with BCECF. Cells stained with rhodamine 123 to visualise mitochondria are shown in the second row. Vital staining of the Golgi by using C_6NBD-ceramide is displayed in the third row and shows a more granular appearance than the mitochondria. Staining of the endoplasmic reticulum (ER) with $DiOC_6$ is shown in the bottom row, the fluorescence again exhibiting a dense granular appearance. The scale bars represent 10 μm.

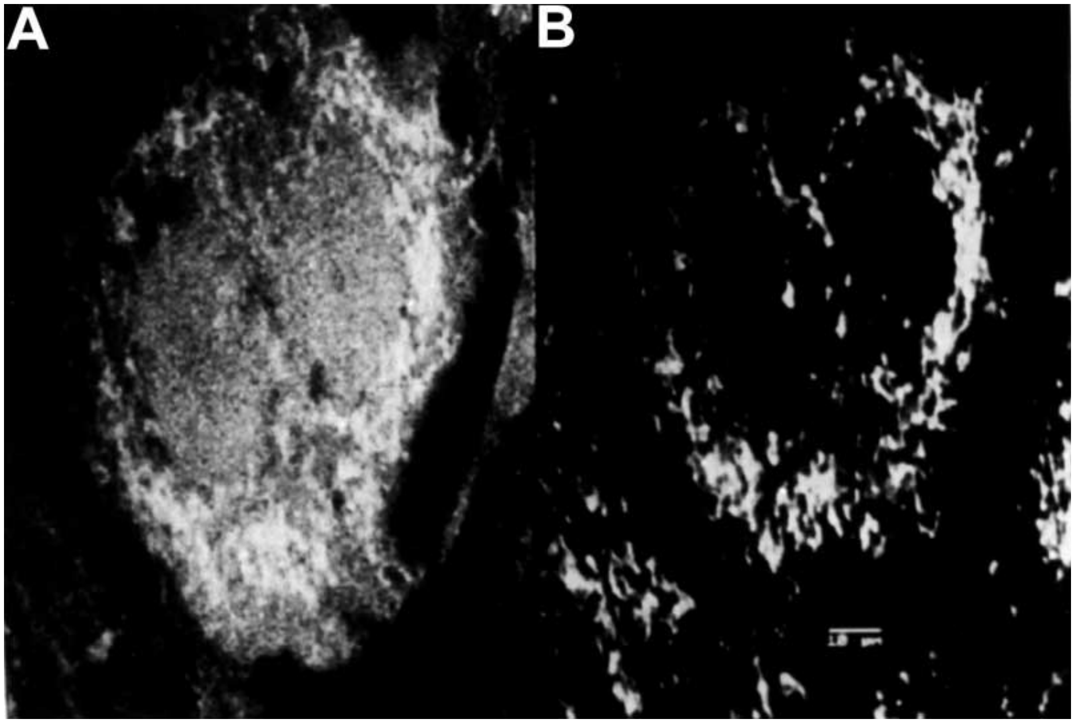

Fig. 2. Confocal laser scanning images demonstrating the co-localization of BCECF staining and mitochondrial staining. (A) Image from TALH cells loaded with BCECF. (B) After photobleaching and staining with rhodamine 123, the level of fluorescence was reduced to less than 10 %, as shown by comparing the fluorescence intensity within the nucleus in A and B. Image analysis on a pixel-by-pixel basis revealed complete coincidence between the rhodamine staining and the BCECF-loaded filamentous structures outside the nucleus. The scale bar in B represents 10 μm.

areas of fluorescence could be distinguished. A very bright and homogeneous fluorescence was always observed in the nucleus, while the cytoplasm showed the lowest level of fluorescence. The difference between the cytoplasm and nucleus was significant in all cases, but was most striking in the endothelial E49 cell line. Within the cytoplasm, some additional inhomogeneity was observed and some ‘dark holes’ with an apparent diameter of approximately 1 μm had barely detectable fluorescence. These probably represent endosomes with a very acidic pH. In addition, all cells contained filamentous structures that showed a significantly brighter fluorescence than the cytoplasm. In LLC-PK_1 cells, the fluorescence of these filamentous structures was similar to that of the nucleus, whereas in the other two cell lines they showed a level of fluorescence between that of the nucleus and the cytoplasm (see Table 1).

Vital staining of cellular organelles

Fig. 1 shows the cellular distribution of mitochondria stained with rhodamine 123 (second row), the Golgi apparatus stained with C_6NBD (third row) and the endoplasmic reticulum DiOC_6 (fourth row). The mitochondria were mainly located around the nucleus and showed a distinct filamentous pattern, in contrast to the Golgi apparatus and endoplasmic reticulum which had a granular appearance. The diameter of the granules was approximately 1 μm for the Golgi and 1.4 μm for the endoplasmic reticulum.

Table 1. *Fluorescence intensities of BCECF in arbitrary units measured with 442 nm excitation (pH-independent wavelength) and emission above 515 nm*

Cell type	Nucleus	Filamentous structures	Cytoplasm
TALH	154±9*	135±6*	124±8
LLC-PK_1	113±26*	122±22*	75±6
E49	168±30*	130±25*	98±23

Values are means ± S.E.M., *N*=4.
*Significantly different from the value for cytoplasm, $P<0.05$.

Direct evidence for a co-localization of BCECF and rhodamine 123

The similarity between the intracellular distribution of mitochondria and the filamentous staining observed with BCECF suggested an association between the two. In order to prove such a connection directly, TALH cells were first loaded with BCECF and a confocal image was obtained. The dye in the cells was then photobleached by scanning the cells with the laser beam until no further reduction of fluorescence was observed. This reduced the intensity in every cell compartment to less than 10 % of the intensity reached prior to photobleaching. This can be seen in Fig. 2 by comparing the fluorescence intensity of BCECF in the nucleus before and after photobleaching. Cells were then superfused with a rhodamine-containing solution to stain the mitochondria, and a confocal image of the same cells was recorded for comparison with the BCECF image. In Fig. 2, image analysis on a pixel-by-pixel basis revealed a complete coincidence between the rhodamine staining and the filamentous BCECF fluorescence located outside the nucleus.

pH measurements

Table 2 gives the steady-state pH values for the various intracellular locations calculated from the image ratio

Table 2. *Steady-state intracellular pH measurement of cells loaded with BCECF*

Cell type	Nucleus	Mitochondria	Cytoplasm
TALH	6.91±0.08	6.96±0.09	6.98±0.10
LLC-PK$_1$	7.28±0.02*	7.40±0.07*	7.36±0.03*
E49	6.84±0.13	6.83±0.13	6.84±0.14

Values are means ± S.E.M., *N*=4.

*Significantly different from the pH value of TALH and E49 cells, $P<0.05$.

488 nm/442 nm and the individual calibration curves. It is evident that there were distinct differences in the cytoplasmic pH between the cell lines, with LLC-PK$_1$ cells showing a significantly higher cytoplasmic pH ($P<0.05$) than the TALH and E49 cells. When the pH values within the different compartments of a single cell type were compared, however, no differences between the nucleus, the mitochondria and the cytoplasm were observed. When the cells were briefly exposed to cyanide (data not shown), which should decrease intramitochondrial pH, no differences could be detected. In addition, as shown in Fig. 3, a pulse of NH_4Cl elicited the same initial alkalization and an identical subsequent acidification, irrespective of the position of the optical field within the cell.

These results strongly suggest that the intracellular BCECF, despite its inhomogeneous distribution, responds uniformly under steady-state conditions and during intracellular alkalosis and acidosis.

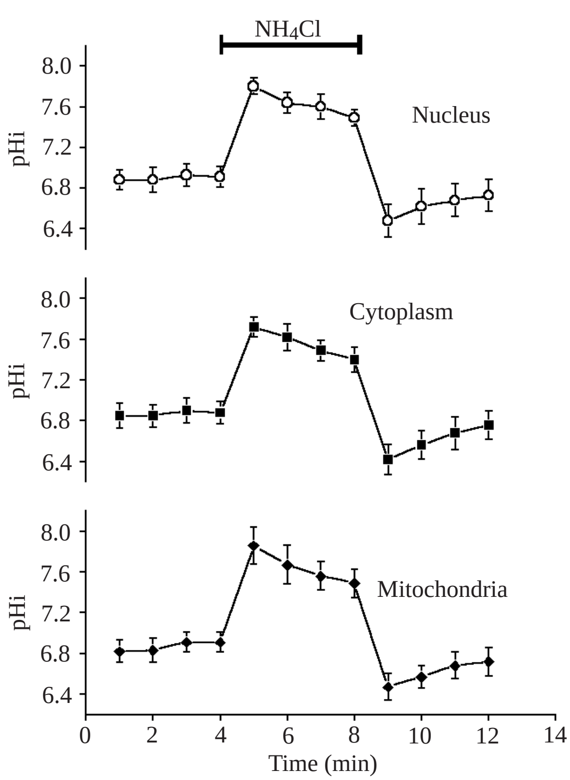

Fig. 3. The effect of a 4 min exposure to 20 mmol l^{-1} NH_4Cl on the intracellular pH recorded in three different cell compartments, the nucleus, the cytoplasm and the mitochondria, in E49 cells. Values are means ± S.E.M., *N*=4.

Discussion

The current investigations extend and refine prior observations in which video microscopy was employed to measure intracellular parameters by the use of fluorescence indicator dyes. Such studies have revealed a relatively high fluorescence over the nucleus compared with that in the cytoplasm (Rosenberg *et al.* 1991). Similarly, relatively high levels of fluorescence have been reported in the nucleus in confocal laser scanning microscopic studies in renal principal cells (Wang and Kurtz, 1990) and in various other cell types (for a review, see Weinlich *et al.* 1994). In addition, some studies revealed a fine granulation that could not be further resolved owing to the limited resolution of the technique (Slayman and Moussatos, 1994). In Swiss 3T3 cells, such punctuate fluorescence was attributed to accumulation of dye in mitochondria (Bright *et al.* 1989).

The level of fluorescence in the nucleus of all the cell types studied was significantly higher than in the cytoplasm. Since BCECF is rather homogeneously distributed within the nucleus, adsorption to the nuclear envelope appears to be unlikely, but whether there is an active intranuclear accumulation or a passive binding to intranuclear components (histones or DNA or both) remains to be established. The observation that the nuclear fluorescence decreases when the viability of the cells is impaired (Weinlich *et al.* 1994) points to metabolic involvement in this phenomenon, and it is possible that ATP-dependent pumps, such as those described in the plasma membranes (Allen *et al.* 1990; Molenaar *et al.* 1992), might be involved. The parallel behaviour of the pH inside the nucleus and the cytoplasm and the similarity in their calibration characteristics suggest that the indicator is only loosely associated with intranuclear components since shifts in the calibration curve would be expected if this were not the case (Owen, 1992; Paradiso *et al.* 1986). Furthermore, protons appear to be able to cross the nuclear envelope easily, leading to rapid equilibration between the intra- and extranuclear spaces.

Our fluorescence measurements from mitochondria indicate that the dye is probably not present in the intramitochondrial matrix space. This is suggested by the following. (1) The steady-state pH found in mitochondria was not more alkaline than that of the cytoplasm, which was unexpected in view of the determination of intramitochondrial pH by other investigators, who have demonstrated pH differences as great as 1 pH unit between mitochondria and cytoplasm (Schoolwerth *et al.* 1989; Thomas *et al.* 1982). (2) The application of cyanide did not alter the mitochondrial pH, although cyanide would be expected to decrease the pH because it inhibits mitochondrial respiration. Third, the pH calculated for the mitochondrial structures was always identical to that of the cytoplasm. The most likely conclusion, therefore, is that BCECF is not inside the mitochondria but is instead associated with their outer compartments or membranes, where it can easily report the proton concentration and its changes in the adjacent cytoplasm.

It is interesting to note that Fura-2, an intracellular [Ca^{2+}] indicator, has also been reported to accumulate in mitochondria (Malgaroli *et al.* 1987; Steinberg *et al.* 1987; Wahl *et al.* 1992). Since this indicator, like BCECF, is negatively charged in its free form, its location within mitochondria remains to be established. Using conventional fluorescence microscopy, investigations on human fibroblasts loaded with Fura-2 revealed a brighter fluorescence in the nucleus than in the cytoplasm (Wahl *et al.* 1992), similar to the situation found with BCECF. In contrast to an even pH distribution throughout the cells, the Fura-2-loaded cells reveal a [Ca^{2+}] gradient between the nucleus and the cytoplasm. This indicates that for experimental measurements of Ca^{2+} it might be necessary to perform subcellular imaging.

In conclusion, the current studies demonstrate directly that in several cell lines BCECF fluorescence only detects changes in cytoplasmic pH, the pH values in other cellular organelles are not recorded. They also show the usefulness of dual-wavelength confocal laser scanning microscopy for optical measurements in small cellular compartments.

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