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# Mitochondrial pH Monitored by a New Engineered Green Fluorescent Protein Mutant*

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**We here describe a new molecularly engineered green fluorescent protein chimera that shows a high sensitivity to pH in the alkaline range. This probe was named mtAlpHi, for mitochondrial alkaline pH indicator, and possesses several key properties that render it optimal for studying the dynamics of mitochondrial matrix pH, *e.g.* it has an apparent p$K_a$ (p$K_a'$) around 8.5, it shows reversible and large changes in fluorescence in response to changes in pH (both *in vitro* and in intact cells), and it is selectively targeted to the mitochondrial matrix. Using mtAlpHi we could monitor pH changes that occur in the mitochondrial matrix in a variety of situations, *e.g.* treatment with uncouplers or $Ca^{2+}$ ionophores, addition of drugs that interfere with ATP synthesis or electron flow in the respiratory chain, weak bases or acids, and receptor activation. We observed heterogeneous pH increases in the mitochondrial matrix during $Ca^{2+}$ accumulation by this organelle. Finally, we demonstrate that $Ca^{2+}$ mobilization from internal stores induced by ionomycin and A23187 cause a dramatic acidification of the mitochondrial matrix.**

In recent years, mitochondria have been the focus of renewed attention by cell biologists. Not only are they pivotal in cell energy metabolism, but they are also involved in other phenomena of key importance, primarily in the control of $Ca^{2+}$ homeostasis and in apoptosis. For example, mitochondrial $Ca^{2+}$ uptake can modulate the kinetics of cytosolic $Ca^{2+}$ changes, and in turn, mitochondrial metabolism is directly controlled by $Ca^{2+}$ uptake, since an elevation of mitochondrial $[Ca^{2+}]_m$[1] activates three key mitochondrial dehydrogenases, thereby increasing NAD(P)H production, electron transport, $H^+$ extrusion, and ATP synthesis. Alterations in mitochondrial pH or membrane potential ($\Delta\psi$), or both, are believed to be essential in the regulation of the so called "permeability transition pore" and thus in controlling apoptosis. (For recent reviews, see Refs. 1–5). While $\Delta\psi$ can now be monitored *in situ* with probes that show high specificity and sensitivity, the methodologies for measuring the pH of the mitochondrial matrix are less evolved. Matrix pH can be monitored in intact cells with pH indicators such as fluorescein, carboxy-SNARF and BCECF, preferentially trapped within mitochondria by manipulating the experimental conditions, *e.g.* temperature, loading, and post-loading incubation times, and dye concentration (6–8). However, the selectivity of such subcellular localization is not very satisfactory and/or the p$K_a$ of the indicators is not optimal for the determination of pH in the mitochondrial matrix. New pH indicators have been synthesized more recently, which are mutants of the widely used green fluorescent protein (GFP); these are genetically encoded molecules, capable of being selectively targeted to different cellular compartments (9–15). Whereas the wild type protein is rather insensitive to pH changes in the physiological range, the fluorescence of some of its engineered mutants is very sensitive to pH changes (9, 13–19). Most of the pH-sensitive GFP mutants available to date, however, have p$K_a$ = 7.1, far from ideal to measure the alkaline environment of the mitochondrial matrix (9, 15, 17–19). Two exceptions, *i.e.* GFP mutants with a higher p$K_a$ (8.0), have been described in recent years (11, 13, 14, 16, 20), but the lack of extensive biological characterization, as well as the suspected problems in folding and/or illumination close to the UV region (13, 14), led us to adopt a new strategy in the development of a pH indicator suited for the mitochondrial matrix.

We here describe the construction and characterization of a new GFP based pH indicator with a p$K_a'$ in the alkaline region (*i.e.* with an ideal sensitivity to monitor pH variations within the mitochondrial matrix), which we named mtAlpHi, for mitochondrial alkaline pH indicator. This probe possesses several properties essential for studying the dynamics of mitochondrial matrix pH; it responds rapidly and reversibly to changes in pH (both *in vitro* and in intact cells), it has an apparent p$K_a$ (p$K_a'$) around 8.5, it is selectively targeted to the mitochondrial matrix, and it lacks toxicity or evident interference with normal cellular functions. Using this novel probe, the dynamics of

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[1] The abbreviations used are: $[Ca^{2+}]m$, mitochondrial matrix, free $Ca^{2+}$ concentration; GFP, green fluorescent protein; mtAlpHi, mitochondrial alkaline pH indicator; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; mtECFP, mitochondrial enhanced cyan fluorescent protein; mtEYFP, enhanced yellow fluorescent protein; FCCP, carbonyl cyanide 4-trifluoromethoxyphenylhydrazone; TPEN, tetrakis-(2-pyridylmethylethyl); TBHQ, 2,5-di-tertbutylhydrochinone; KRB, Krebs-Ringer buffer; BAPTA-AM, 1,2-bis(2-aminophenoxy)ehtane-*N*,*N*,*N*′,*N*′-tetraacetic acid acetoxymethyl ester; SERCA, sarco-endoplasmic reticulum calcium ATPase.

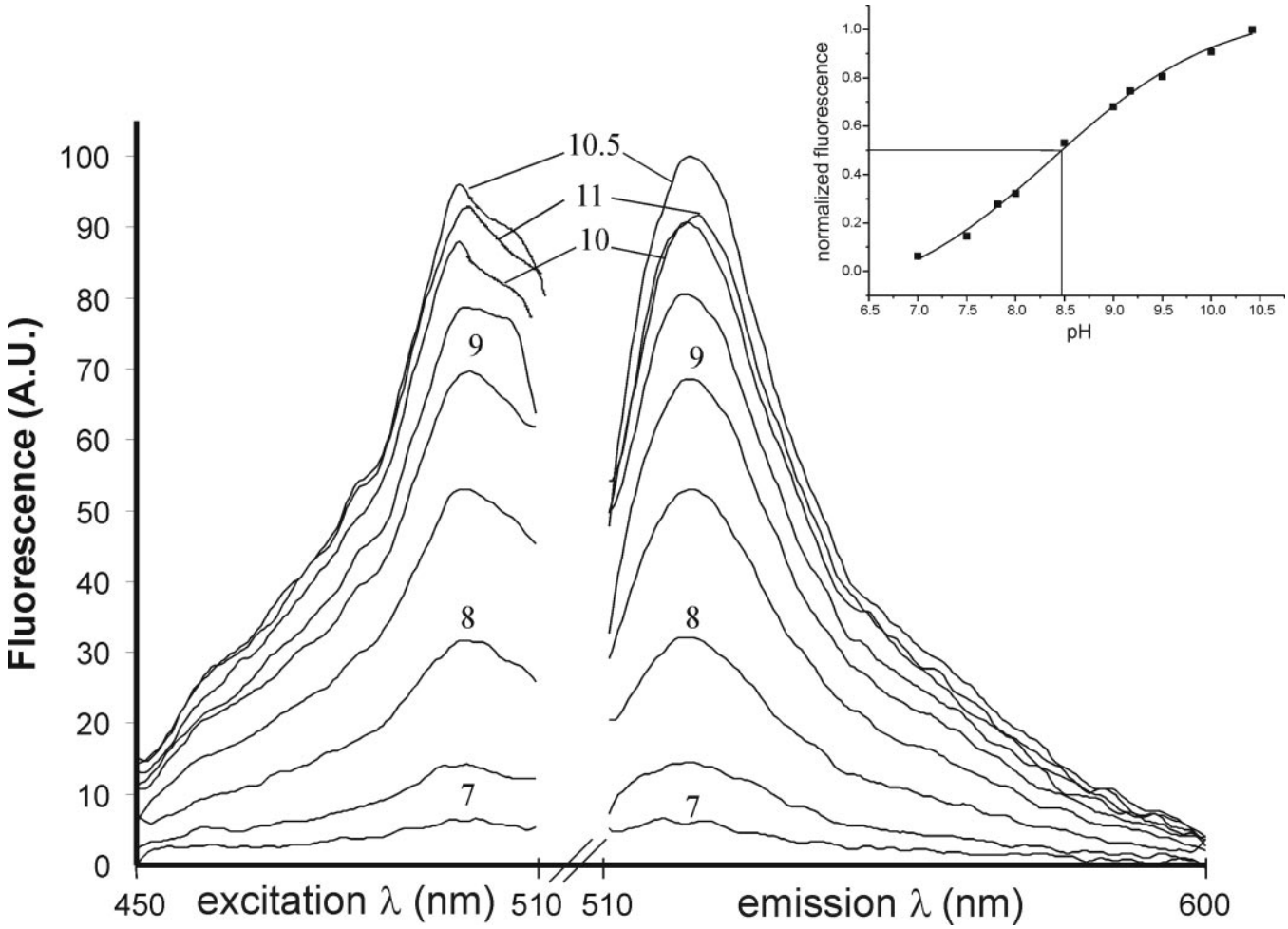


FIG. 1. ***In vitro* pH-dependent fluorescence intensity of mtAlpHi.** The fluorescence intensity of the purified protein was measured as a function of pH, ranging from 7 to 11 (every 0.5 unit). For the *traces* on the *left part* of the figure, samples were scanned from 450 to 510 nm and the fluorescence intensity monitored at 522 nm; for the traces on the *right part* of the figure, samples were excited at 498 nm and fluorescence monitored from 510 to 600 nm. In the *inset*, normalized peak fluorescence intensities (excitation 498 nm, emission 522 nm) are plotted against pH; from this graph, the apparent p$K_a$ of mtAlpHi is ~8.5.

mitochondrial pH were monitored *in situ* under different experimental conditions, and new information concerning this key parameter was obtained.

## MATERIALS AND METHODS

*Generation of Constructs*—The new pH indicator described in this paper derives from the EYFP based on insertion in the $Ca^{2+}$ indicator camgaroo2 (Ref. 21; kindly provided by R. Y. Tsien), with the substitution of calmodulin by a portion of aequorin. The chosen part of aequorin spans bp 334 to 553 of the wild type cDNA and contains two of three active EF-hands of aequorin; the final construct, mtAlpHi, is mitochondrially targeted. For prokaryotic expression, the mitochondrial targeting sequence was removed and the remaining coding region was transferred to pRSET-B (Invitrogen, Milan, Italy); the expressed recombinant protein possesses an N-terminal polyhistidine tag. Details of all constructs are available upon request.

*Protein Expression and in Vitro pH Sensitivity*—For *in vitro* analyses, the recombinant protein with a polyhistidine tag was expressed in *Escherichia coli* BL21(DE3) pLysS (Invitrogen). Bacteria were lysed by sonication, cell debris were removed by centrifugation, and the soluble protein was purified from the supernatant by affinity chromatography using a nickel-coated resin (Ni-NTA Spin Kit; Qiagen, Milan, Italy). Protein was quantified by the Bradford method, using bovine serum albumin as standard. A PerkinElmer Life Science LS55B fluorescence spectrometer was used to determine the excitation and emission spectra of the purified protein (0.3 mg/ml in 50 mM $NaH_2PO_4$, 300 mM NaCl, 250 mM imidazole, pH 8), as well as the dependence of its fluorescence intensity on calcium and pH; the fluorescence sensitivity to [$Ca^{2+}$] was tested from $10^{-9}$ to $10^{-2}$ M, whereas the pH was varied from 7.0 to 11.0.

*Mammalian Cell Expression*—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% $CO_2$. For transient expression, cells were seeded onto 24-mm diameter round glass coverslips and transfections were performed at 50–70% confluence with the calcium phosphate method, using a total of 8 $\mu$g of DNA; for co-transfections, the ratio between the two constructs was 50:50.

*Cortical Neuronal Culture*—Mixed cultures of astrocytes and neurons were obtained from rat neonatal cortices as described previously (22). Modifications were introduced to optimize neuronal survival as described in Ref. 23. For transient expression, cortical neurons were seeded onto 24-mm diameter round glass coverslips, and transfections were performed with the LipofectAMINE 2000 method (Invitrogen), using a total of 1.6 $\mu$g of DNA; for co-transfections, the ratio between the two constructs was 1/1.

*Fluorescence Microscopy*—Cells expressing the fluorescent probes were observed 48 h after transfection on an inverted fluorescence microscope (Zeiss Axioplan), a Fluar® oil immersion objective (40×, N.A. 1.30) was used. Excitation light at appropriate wavelengths was produced by a monochromator (Polychrome II, TILL Photonics, Martinsried, Germany): 425 nm for mtECFP, 480 nm for mtAlpHi, and 510 nm for mtEYFP. For cells loaded with fura-2, excitation was at 340 and 380 nm; emitted light was collected through a FT425 dichroic beamsplitter and a 480EFLP emission filter. For cells co-transfected with mtAlpHi and mtECFP, a 505DRLP dichroic beamsplitter was used, and emitted light was collected through a 535RDF45 emission filter. For cells co-transfected with mtEYFP and mtECFP cells, a JP4 BS dichroic beamsplitter was used, and emitted light was collected through a 51017M + 10 (CFP/YFP dual) emission filter. Unless otherwise stated, filters and dichroic beamsplitters were purchased from Omega Optical and

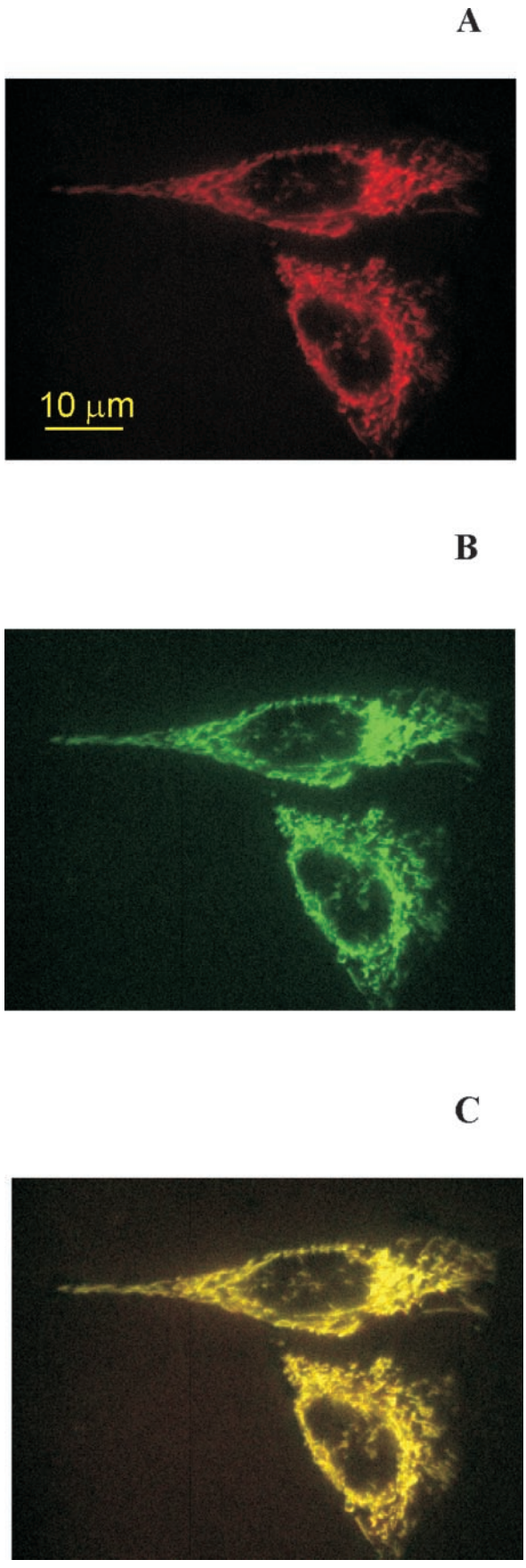


FIG. 2. **Subcellular localization of mtAlpHi.** HeLa cells were co-transfected with mtAlpHi and mtECFP; the two probes were selectively excited at 480 nm (*A*) and 425 nm (*B*), respectively. Overlay of the two images reveals a perfect co-localization of the two probes (*C*), demonstrating the mitochondrial localization of mtAlpHi.

Chroma Technologies (Brattleboro, VT). Images were acquired with a cooled CCD camera (Imago, TILL Photonics) attached to a 12-bit frame rabber. 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.

*Calcium Measurements with fura-2*—HeLa cells were loaded with the cell permeant probe fura-2 AM (2 $\mu$M) for 30 min at 37 °C, in a Krebs-Ringer buffer (KRB; containing, in mM, 125 NaCl, 5 KCl, 1 $Na_3PO_4$, 1 $MgS0_4$, 1 $CaCl_2$, 5.5 glucose, 20 Hepes, pH 7.4 at 37 °C). Cells were washed and incubated in KRB for another 20 min at room temperature, to complete desterification, before measurements were started.

*Aequorin Measurements*—Cells transiently expressing aequorin were reconstituted in KRB, supplemented with 1 mM $CaCl_2$, and 5 $\mu$M coelenterazine for 1 h. The cells were transfected with either the cDNA encoding mtaequorin or co-transfected with mtaequorin and mtAlpHi. During the experimental procedure, cells were placed in a temperature-controlled chamber, at 37 °C, and perfused with KRB. Photons emitted were collected and analyzed as described previously (24).

*Cell Stimulations*—For *in situ* calibration experiments, HeLa cells were perfused with modified KRB (containing, in mM, 5 NaCl, 125 KCl, 1 $Na_3PO_4$, 1 $MgS0_4$, 10 Hepes for pH 6–7.5, in 0.5 steps; for pH values 8 and above, Hepes was substituted by Tris). All calibration experiments were carried out in the presence of nigericin and monensin (5 $\mu$M each). KRB was used for experiments with sodium acetate and $NH_4Cl$ (30 mM in each case); in parallel, [NaCl] was brought down to 95 mM.

Permeabilization of HeLa cells was carried out by incubating in KRB-3 (containing, in mM, 10 NaCl, 130 KCl, 1 $Na_3PO_4$, 5 succinate, 1 ATP, 1 EGTA, 10 Tris, pH 8.0), supplemented with digitonin (100 $\mu$M). After permeabilization, cells were maintained in KRB-3 with or without $MgCl_2$.

Cortical neurons were perfused with standard solutions as described in Ref. 23. Perfusion with histamine (100 $\mu$M) was employed to trigger $Ca^{2+}$ release from intracellular stores; perfusion with 2,5-di-tert-butylhydrochinone, (TBHQ, 30 $\mu$M) was employed to inhibit endoplasmatic reticulum $Ca^{2+}$-ATPase; ionomycin was employed at 1 $\mu$M and FCCP at 4 $\mu$M. Unless otherwise indicated, all experimental procedures were carried out at room temperature. Coelenterazine, fura-2, BAPTA-AM and tetrakis-(2-pyridylmethylethyl)enediamine (TPEN) were obtained from Molecular Probes (Leiden, The Netherlands); ionomycin was from Calbiochem (La Jolla, CA). All other materials were of analytical or highest available grade and acquired from Sigma (Milan, Italy). All typical experiments presented are representative of at least three trials with very similar results. Numerical data are presented as mean ± S.D.

## RESULTS

*Construction and Properties of mtAlpHi, a New, GFP-based, pH Indicator*—The fluorescence of wild type GFP is notoriously resistant to variations in ambient parameters, including pH, ionic strength, as well as cation and anion concentration. Conversely, most GFP mutants are sensitive to changes in the surrounding milieu, in particular pH. During the characterization of some $Ca^{2+}$-sensitive GFP mutants, we observed that insertional mutants of YFP, the so called "camgaroos," are, unlike YFP itself, quite sensitive to pH changes above 8.[2] We thus undertook the design of a pH indicator suited for measurements within the mitochondrial matrix. As described under "Materials and Methods," we replaced the insertion of camgaroo (calmodulin) with a similar sized peptide. The new insertion molecule is a deleted version of aequorin, comprising 73 amino acids containing the second and the third EF-hand domains, thus lacking the first EF-hand, elimination of this part of aequorin was deemed suitable, since it practically abolishes the $Ca^{2+}$-dependent luminescence of aequorin.[2] Targeting to the mitochondrial matrix was achieved in the same way as in camgaroo (21).

The pH and $Ca^{2+}$ sensitivity of the purified chimeric molecule were tested *in vitro*. Excitation and emission spectra at different pH values are shown in Fig. 1 and are similar to those of the original citrine (21). The fluorescence intensity increased

[2] M. F. Cano Abad, G. Di Benedetto, P. J. Magalhães, L. Filippin, and T. Pozzan, unpublished observations.

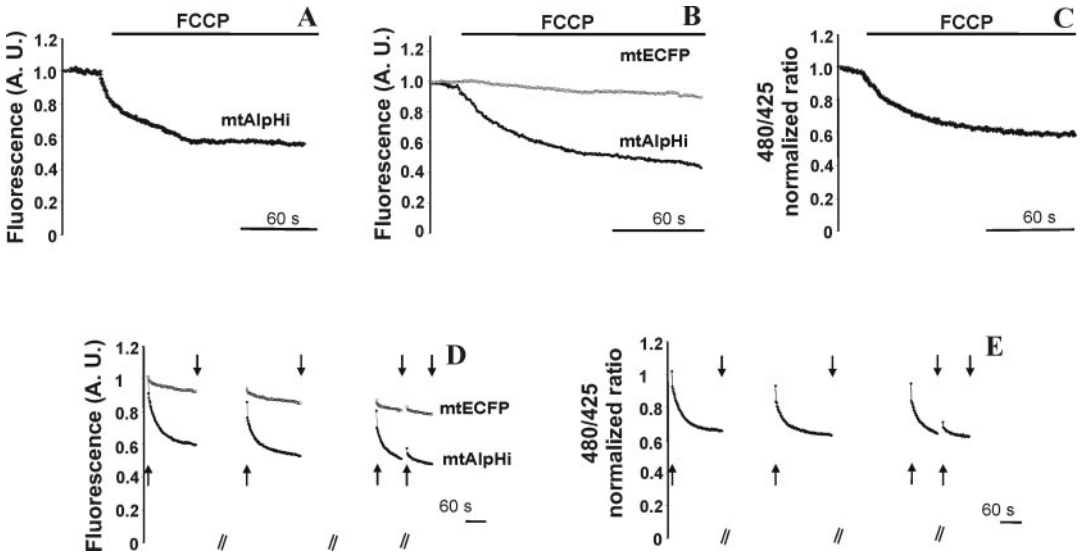


FIG. 3. **Sensitivity of mtAlpHi to uncouplers and illumination conditions.** *A*, HeLa cells transfected with mtAlpHi and treated with FCCP (4 $\mu$M) show a marked drop in fluorescence intensity that flattens into a plateau. *B*, HeLa cells co-transfected with mtAlpHi and mtECFP were treated with FCCP as in *A*; the fluorescence of mtECFP suffers only a marginal decrease due to photobleaching, whereas that of mtAlpHi behaves as in *A*. The fluorescence ratio of the two probes (mtAlpHi/mtECFP) is shown in *C*. *D*, effect of illumination on the individual fluorescence of mtAlpHi and mtECFP ("light on" and "light off" is indicated by ↑ and ↓, respectively). Upon constant illumination, mtAlpHi suffers a rapid photoisomerization that reduces its fluorescence intensity by ~40%; similar illumination does not influence mtECFP markedly (the small reduction is simply due to photobleaching). The reduction in fluorescence due to photoisomerization is recovered after periods of non illumination of at least 60 s; as seen on the *right part* of the panel, if the periods of non-illumination are too brief (<10 s), the recovery is severely compromised. Normalized ratio values of these traces are shown in *E*. In *A–C*, values are normalized to $F_0$, where $F_0$ represents the average of five consecutive images, selected after the fluorescence reached a stable plateau and before any stimuli were applied; in *D* and *E*, $F_0$ is the value of the first image acquired upon illumination.

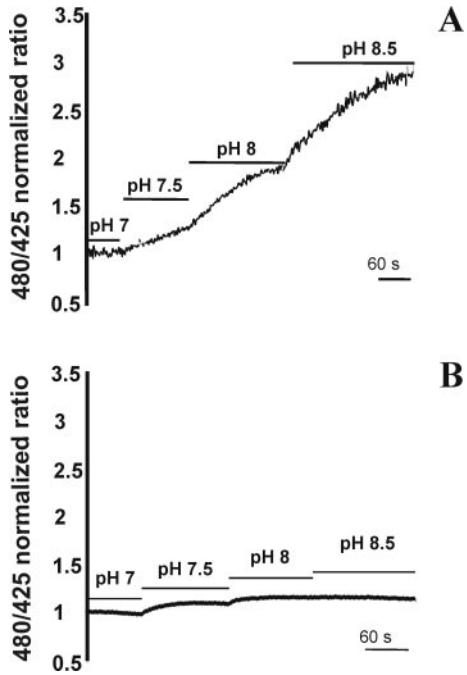


FIG. 4. ***In situ* pH-dependent fluorescence intensity of mtAlpHi.** *A*, HeLa cells co-expressing mtAlpHi and mtECFP were perfused with mKRB, supplemented with the ionophores nigericin and monensin (5 $\mu$M). The pH was increased in 0.5 steps, from 7.0 to 8.5, and the fluorescence of both probes was monitored; the ratio values were calculated as described in the legend to Fig. 3*C*. *B*, For comparison, HeLa cells co-expressing mtEYFP and mtECFP were subjected to the same protocol. Whereas mtAlpHi yields a signal that increases by ~3-fold between pH 7 and 8.5, mtEYFP shows an increase of only <15%.

as pH was raised from 7.0 to 10.5, after which the trend was reversed, most likely due to the denaturation of the protein under these extreme conditions. Plotting normalized peak fluorescence intensities (excitation 498 nm, emission 522 nm) against pH reveals an apparent p$K_a'$ of 8.5 (Fig. 1, *inset*), in a medium mimicking intracellular cation concentration. The $Ca^{2+}$ sensitivity was also tested and no variation in fluorescence was observed for changes in [$Ca^{2+}$] from $10^{-9}$ to $10^{-2}$ M (data not shown).

*mtAlpHi as an in Situ Mitochondrial pH Indicator*—HeLa cells transiently expressing mtAlpHi show a typical mitochondrial pattern (Fig. 2*A*). To confirm the mitochondrial localization of mtAlpHi, we co-transfected cells with a cyan mutant of GFP targeted to the mitochondrial matrix (mtECFP; Fig. 2*B*); as seen in Fig. 2*C*, the two probes show a perfect colocalization.

The effect of a classical protonophore, FCCP, on the fluorescence of mtAlpHi is shown in Fig. 3*A*. As expected, FCCP induced a rapid drop in fluorescence that reached a new steady state within 1–2 min; the rate of the drop depends on the dose of the uncoupler (data not shown). It should be noted that, as shown in Fig. 1, the pH changes result only in changes of the fluorescent intensity of mtAlpHi but not in a spectral shift. Given the different expression levels in different cells and, especially, the continuous changes in mitochondrial shape and position, quantitative measurements with a single, non-ratiometric indicator are prone to artifacts and often difficult to interpret correctly. To bypass this problem, mtAlpHi was co-transfected with mtECFP. The choice of ECFP as a partner for mtAlpHi resides in the fact that both molecules can be imaged simultaneously, being distinguishable in both excitation and emission spectra. In addition, among GFP mutants, ECFP is one of the most stable with respect to pH changes (9). The use of two probes loaded in the same compartment has been introduced in the past to bypass the problem of non-ratiometric $Ca^{2+}$ indicators, and although not devoid of problems, it is a simple way to correct for movement artifacts, change of focus, etc. For a detailed discussion of this point see Refs. 9 and 25.

The effect of an uncoupler on the fluorescence of mtAlpHi and mtECFP co-expressed in the same cell is shown in Fig. 3*B*. Upon addition of FCCP, the fluorescence of mtECFP was practically unaffected, while that of mtAlpHi dropped, as shown

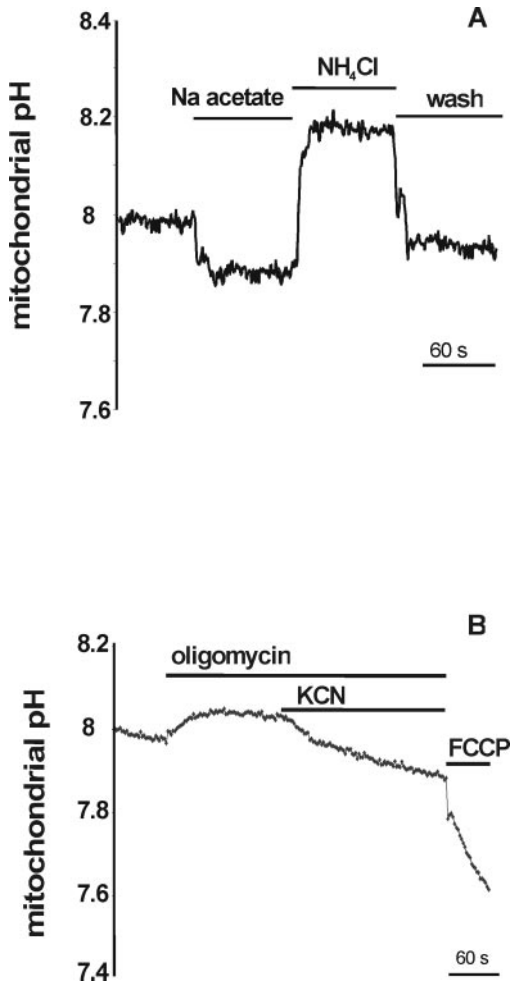


FIG. 5. **Reversibility of the pH-dependent fluorescence intensity of mtAlpHi.** As in Fig. 4*A*, HeLa cells were perfused with sodium acetate (30 mM) and $NH_4Cl$ (30 mM), as indicated. *A*, in the presence of the weak acid, a drop in fluorescence was observed, whereas the weak base provoked a sharp increase in fluorescence. Upon washing, the signal returned to approximately basal levels. *B*, where indicated cells were first perfused with oligomycin (4 $\mu$g/ml), followed by the classic inhibitor of the complex IV, cyanide (3 mM). The final addition of the protonophore FCCP (4 $\mu$M) resulted in a further faster acidification.

before. The fluorescence ratio mtAlpHi/mtECFP is presented in Fig. 3*C*.

The experiment presented in Fig. 3, *D* and *E*, shows an additional characteristic of the fluorescence of mtAlpHi, also found in the YFP from which it derives. Immediately upon illumination, the fluorescence of mtAlpHi drops rapidly, without any added stimulus, until the initial fluorescence is reduced by about 40%; afterward, the fluorescence intensity is characterized by a slowly declining plateau. This initial drop in fluorescence is most likely due to photoisomerization, given that the signal recovers to almost the initial value if the exciting beam is switched off for a few tens of seconds. Brief periods of non-illumination, *e.g.* 5–10 s, were sufficient to promote only a partial recovery (Fig. 3*D*). The declining plateau, on the other hand, is due to photobleaching, in as much as it does not recover after a period of non-illumination. The fluorescence of mtECFP, on the contrary, shows a marginal rapid drop upon start of illumination. The rates of photobleaching are similar for both mtAlpHi and mtECFP. The fluorescence ratio reflects these characteristics, dropping initially and then it is practically constant (Fig. 3*E*).

For the pH calibration of mtAlpHi fluorescence *in situ*, HeLa cells co-expressing mtAlpHi and mtECFP were perfused with high $K^+$ solutions at different pH values, in the presence of nigericin and monensin (to equilibrate extra- and intracellular pH). The increase in pH resulted in a substantial increase in the fluorescence of mtAlpHi, while the signal of mtECFP remained practically unaltered, giving rise to the steep ratio change shown in Fig. 4*A*. For comparison, control cells co-expressing mtEYFP and mtECFP were subjected to the same experimental protocol, yielding a much flatter ratio (Fig. 4*B*). The dynamic range of mtAlpHi fluorescence is markedly different from that of mtEYFP: whereas the former increases ~3-fold between pH 7 and 8.5, the latter shows a much smaller change (<15%). With this calibration, we calculate the mitochondrial matrix pH in intact cells in steady state conditions to be 8.05 ± 0.11; upon addition of FCCP this value drops to about 7.50 ± 0.22.

Finally, two other compounds known to alter mitochondrial pH were applied to cells co-expressing mtAlpHi and mtECFP, *i.e.* sodium acetate and $NH_4Cl$. As expected, addition of sodium acetate caused a net decrease in mitochondrial pH; conversely, addition of $NH_4Cl$ induced an increase in pH; the steady state pH was reached again after washout (Fig. 5*A*).

Additional experiments were carried out to verify the sensitivity of the system to drugs that interfere with ATP synthesis (olygomycin) or the electron flow in the respiratory chain. The block of the ATPase with oligomycin resulted in a modest pH increase, while further addition of cyanide caused a slow but larger drop of pH, as predicted. In parallel we also measured $\Delta\psi$ with the potential sensitive dye TMRM; a small increase was observed upon addition of oligomycin, while a slow collapse of $\Delta\psi$ occurred upon further addition of KCN (data not shown).

Although GFP is considered rather inert in terms of cell physiology, the possibility that the construct may interfere with cellular functions must be taken into consideration. In particular, since mtAlpHi contains two EF-hand ($Ca^{2+}$-binding domains), its effect on $Ca^{2+}$ handling in the cytosol and mitochondria was tested. To determine cytosolic variations in $[Ca^{2+}]$ ($[Ca^{2+}]_c$), cells expressing mtAlpHi were loaded with the ratiometric calcium indicator fura-2 and challenged both with histamine and 2,5-di-*tert*-butylhydrochinone (TBHQ), two well characterized agents that raise $[Ca^{2+}]_c$. The changes in fura-2 signal were monitored, and although there was some variability in the response among individual cells, there was practically no difference between control and mtAlpHi expressing cells (Fig. 6). To verify that mitochondrial $Ca^{2+}$ handling is also unaffected by expression of mtAlpHi, cells were co-transfected with the pH indicator and with aequorin targeted to mitochondria (mtaequorin; Ref. 26). As shown in Fig. 7, mitochondrial $Ca^{2+}$ handling is unaffected by the expression of mtAlpHi.

*Mitochondrial pH Changes in Response to $Ca^{2+}$ Mobilizing Agents*—The uptake of $Ca^{2+}$ by mitochondria is linked to the extrusion of $H^+$ from the matrix. In isolated mitochondria, $Ca^{2+}$ uptake is known to cause a drastic pH alkalinization if no permeant anions are present in the medium (for a recent review, see Ref. 27). The effect of $Ca^{2+}$ uptake in mitochondria of intact cells was thus tested. HeLa cells co-transfected with mtAlpHi and mtECFP were challenged with histamine and the pH response monitored. We found that only ~14% (25/184) of the cells analyzed responded to histamine and in this case with a small and reversible pH increment (Fig. 8*A*, *black trace*); the vast majority of cells did not show any variation in fluorescence (Fig. 8*A*, *gray trace*). Other protocols to induce more prolonged $Ca^{2+}$ increases in the mitochondria were also tested, *e.g.* the application of TBHQ in $Ca^{2+}$-free medium, followed by re-addition of $Ca^{2+}$; no significant changes in pH were observed under these conditions (data not shown).

To verify whether pH changes within the matrix occur under

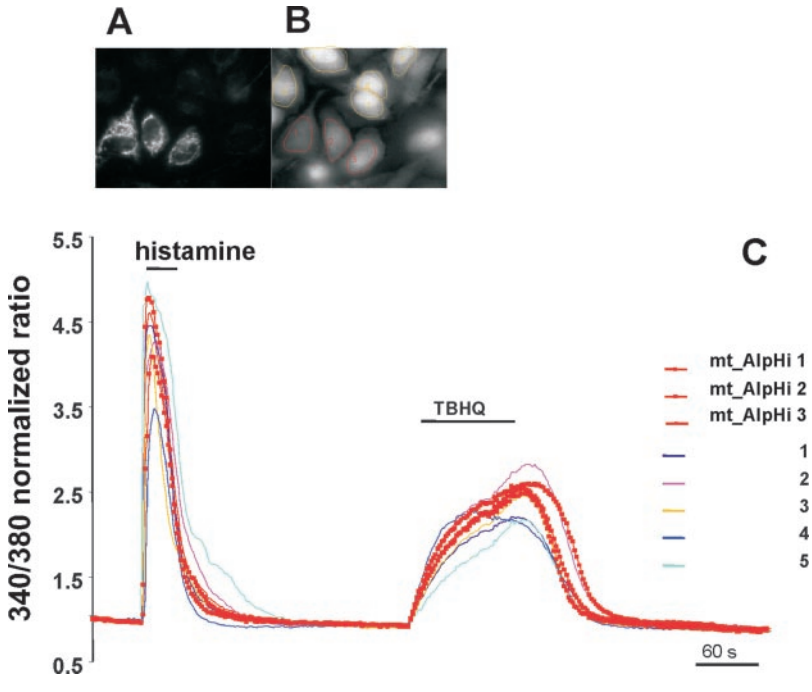


FIG. 6. **Normal cytosolic $Ca^{2+}$ handling by HeLa cells expressing mtAlpHi.** HeLa cells transfected with mtAlpHi were loaded with fura-2. *A*, cells expressing mtAlpHi, identified as described in the legend to Fig. 2. *B*, the same field, visualized with standard fura-2 setting (excitation 340 nm); cells expressing (*thick red traces*) and not expressing (*thin multicolored traces*) mtAlpHi were selected for analysis. *C*, fura-2 signal intensity of the selected regions in *B*. Perfusion with histamine (100 $\mu$M; 30 s) and TBHQ (30 $\mu$M; 2 min) showed no differences between the two types of cells, demonstrating that mtAlpHi does not influence the $Ca^{2+}$ handling abilities of the cell.

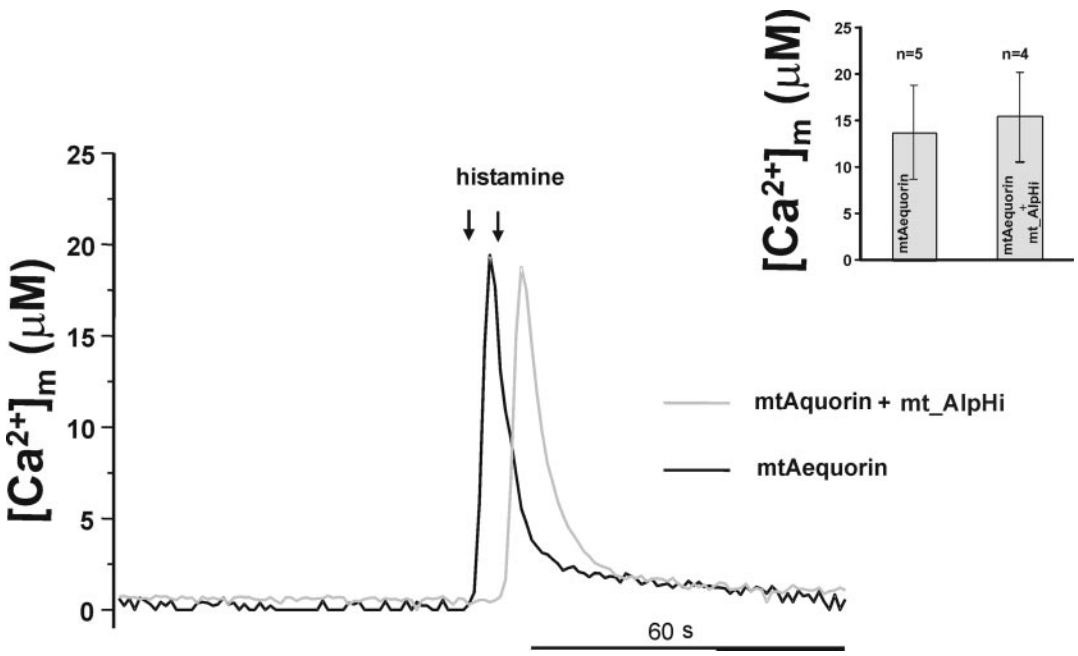


FIG. 7. **Normal mitochondrial $Ca^{2+}$ handling by HeLa cells expressing mtAlpHi.** HeLa cells were co-transfected with mtAlpHi and mtAequorin and challenged with histamine (100 $\mu$M); as controls, HeLa cells expressing mtAequorin alone were challenged in the same way. The *traces* show a similar response in both cases. In the *inset*, it can be seen that the peak amplitude of mitochondrial $Ca^{2+}$ uptake is the same in both conditions.

conditions more relevant for physiology or pathology, experiments were carried out in primary cultures of cortical neurons treated with the excitatory neurotransmitter glutamate. Upon treatment with glutamate, a rapid substantial alkalinization was observed in three out eight neurones analyzed. A typical example of the kinetics of this pH change in a single neuron is presented Fig. 8*B*.

A common protocol to cause the release of $Ca^{2+}$ from internal stores is that of adding a $Ca^{2+}$ ionophore, such as ionomycin or A23187, to cells incubated in $Ca^{2+}$-free medium. The ionophores penetrate into the cells and catalyze the transport of $Ca^{2+}$ across internal membranes, down its electrochemical gradient. Because these ionophores transport $Ca^{2+}$ in exchange with $2H^+$, and $Ca^{2+}$ uptake by mitochondria may result in alkalinization of the matrix, the possibility that these drugs may alter the pH of the organelles was considered. HeLa cells co-expressing mtAlpHi and mtECFP were treated with ionomycin in a $Ca^{2+}$-free solution, containing EGTA. Under these conditions there was a rapid and transient increase in cytosolic $Ca^{2+}$, as measured with fura-2 (not shown). Fig. 9*A* shows that ionomycin induced a pronounced and prolonged drop in mitochondrial pH. Such a result was unexpected, given that one would have predicted, if anything, an alkalinization due to mitochondrial $Ca^{2+}$ uptake and $H^+$ extrusion. Such changes in mitochondrial pH (*i.e.* net alkalinization) were indeed obtained if massive $Ca^{2+}$ accumulation in the mitochondria were induced by incubating the cells with high concentration of ionomycin and $Ca^{2+}$ in the medium (data not shown).

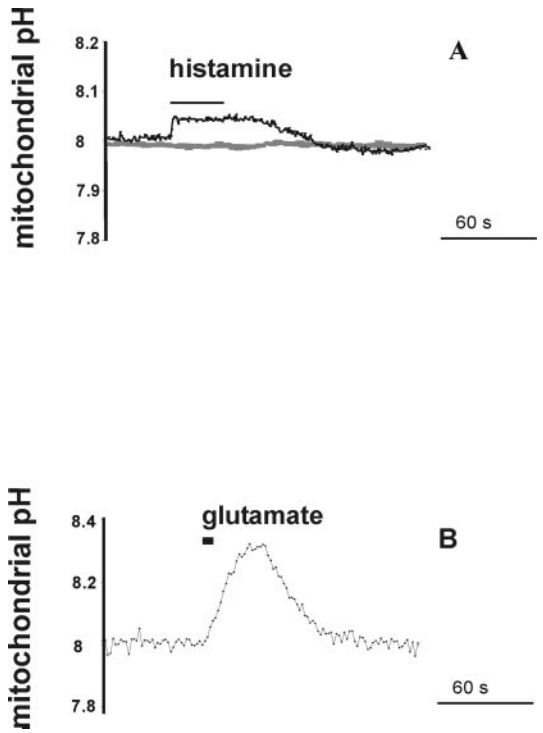


FIG. 8. **pH variations induced by mitochondrial calcium uptake.** *A*, HeLa cells co-expressing mtAlpHi and mtECFP were challenged with histamine (100 $\mu$M, 30 s). In a minority of cases, cells responded with a small increase in mitochondrial matrix pH (as in *black trace*), but most cells did not show any response (as in the *gray trace*). *B*, a typical response of a cortical neuron co-expressing mtAlpHi and mtECFP stimulated with glutamate (100 $\mu$M, for 10 s). An alkalization of the mitochondrial matrix was observed in three out of eight neurons analyzed.

To verify whether or not the changes in mitochondrial pH depend on the mobilization of $Ca^{2+}$ from the endoplasmatic reticulum, the cells were incubated in $Ca^{2+}$-free medium plus EGTA and treated with a SERCA inhibitor. Under these conditions, endoplasmatic reticulum $Ca^{2+}$ is released and the addition of ionomycin results in a negligible further increase in cytoplasmic $[Ca^{2+}]_c$ (not shown). The effect of ionomycin on matrix pH was identical to that under control conditions, *i.e.* without SERCA pump inhibition (data not shown).

Two possible explanations could be offered for this unexpected acidification; (i) ionomycin transports $Ca^{2+}$ out of the mitochondrial matrix in exchange for $H^+$; (ii) the ionophore transports other cations out of the matrix in exchange for $H^+$. To directly test those hypotesis the following experiment was carried out, HeLa cells were loaded with the $Ca^{2+}$ chelator BAPTA in $Ca^{2+}$-free medium containing 1 mM EGTA. These loading conditions cause a decrease in $[Ca^{2+}]$ to unmeasurably low levels (around 10 nM) not only of cytosolic $Ca^{2+}$ but also of $Ca^{2+}$ within organelles (not shown and see Ref. 28). Fig. 9*B* shows that also under these conditions ionomycin still caused the typical drop in pH. The second possibility was tested in the next experiments. We first verified the possibility that heavy metals, such as $Zn^{2+}$ and $Cu^{2+}$ (known to be transported by ionomycin), could be released in exchange for $H^+$. To test this hypothesis we employed the high affinity, membrane-permeable, heavy metal chelator TPEN. This drug is known to penetrate all cell membranes and to chelate with very high affinity most heavy metals (29). The presence or absence of TPEN, however, did not cause any significant difference in the pH effect of ionomycin (data not shown).

Although ionomycin is considered highly selective for $Ca^{2+}$, it can potentially transport $Mg^{2+}$ as well but with lower affinity. We thus considered the possibility that the paradoxical pH acidification caused by the ionophore could be due to $Mg^{2+}$ transport. Indeed, the $Mg^{2+}$ concentration in the cytoplasm is about 1 mM, and a similar concentration is believed to exist in the mitochondrial matrix. Thus, at least in theory, ionomycin could transport $Mg^{2+}$ out of the matrix, driven by the pH difference across the inner mitochondrial membrane. Were this the case, one would expect that by altering the pH or the $Mg^{2+}$ gradients between cytoplasm and mitochondrial matrix should abolish this effect of the ionophore. Acidification of matrix pH (caused by pretreatment with FCCP) prevented any further effect of ionomycin (Fig. 9*C*).

More complex is to alter the intracellular $Mg^{2+}$ gradients, given that changes in cellular $Mg^{2+}$ concentrations are very slow to occur in intact cells (26). We thus employed the following protocol; HeLa cells co-transfected with mtAlpHi and mtECFP were first permeabilized with digitonin, in a $Ca^{2+}$- and $Mg^{2+}$-free solution containing ATP and an oxidizable substrate, succinate. Under these conditions, addition of ionomycin caused again a drop in pH (Fig. 9*D*). Two consecutive additions of 10 and 20 mM $MgCl_2$, in the continuous presence of ionomycin, resulted in the net alkalinization of the matrix, revealing that the pH gradient can indeed drive a ionomycin-catalyzed $Mg^{2+}/2H^+$ exchanges. Similar experiments were carried out with A23187, and the results were qualitatively identical to those obtained with ionomycin.

In conclusion, the common protocol employed for emptying intracellular $Ca^{2+}$ stores with $Ca^{2+}$ ionophores not only causes the release of the $Ca^{2+}$ from these deposits but can also cause a net acidification of mitochondrial pH, an unexpected effect, possibly dependent on the release of $Mg^{2+}$ from the matrix.

## DISCUSSION

The $H^+$ gradient across the inner mitochondrial membrane generated by the respiratory chain, $\Delta\mu H$, is the sum of an electrical component, the membrane potential, $\Delta\psi$, and a concentration component, $\Delta$pH. Measurement of $\Delta\mu H$ is essential to understand the energetic status of the organelles and over the last few years many approaches to monitor this parameter in living cells have been described. The novel GFP-based pH indicator mtAlpHi described here appears to fulfil many of the necessary requirements to be employed as a useful mitochondrial pH probe; (i) it is highly fluorescent and selectively targeted to the matrix; (ii) its apparent $pK_a'$ is around 8.5; (iii) it shows large fluorescence changes between 7 and 9; (iv) it does not interfere with either cytoplasmic or mitochondrial $Ca^{2+}$ handling.

Given that mtAlpHi undergoes changes only in fluorescent intensity upon pH changes, it is prone to the potential artifacts of all non-ratiometric indicators. However, the co-transfection with mtECFP largely circumvents this drawback.

The dynamic range of mtAlpHi within the mitochondrial matrix is similar to that in free solution and is significally better than YFP in the typical pH range of the mitochondrial matrix. Through the use of mtAlpHi we have been able to demonstrate that the $Ca^{2+}$ accumulation induced in mitochondria of HeLa cells by treatment with a $Ca^{2+}$ mobilizing agonist such as histamine results in a subpopulation of cells in a small, but measurable, increase in matrix pH. Considering that the increases in mitochondrial $Ca^{2+}$ concentration in these cells can reach several tens of micromolar (4, 30–32), the small size of this alkalinization and the fact that it occurs only in a subpopulation of cells indicates that $H^+$ extrusion occurring in mitochondria of living cells is well compensated by the pH buffering capacity of the matrix and/or by the movement of anions, most likely phosphate, across the inner membrane. Less pronounced, but more prolonged uptake of

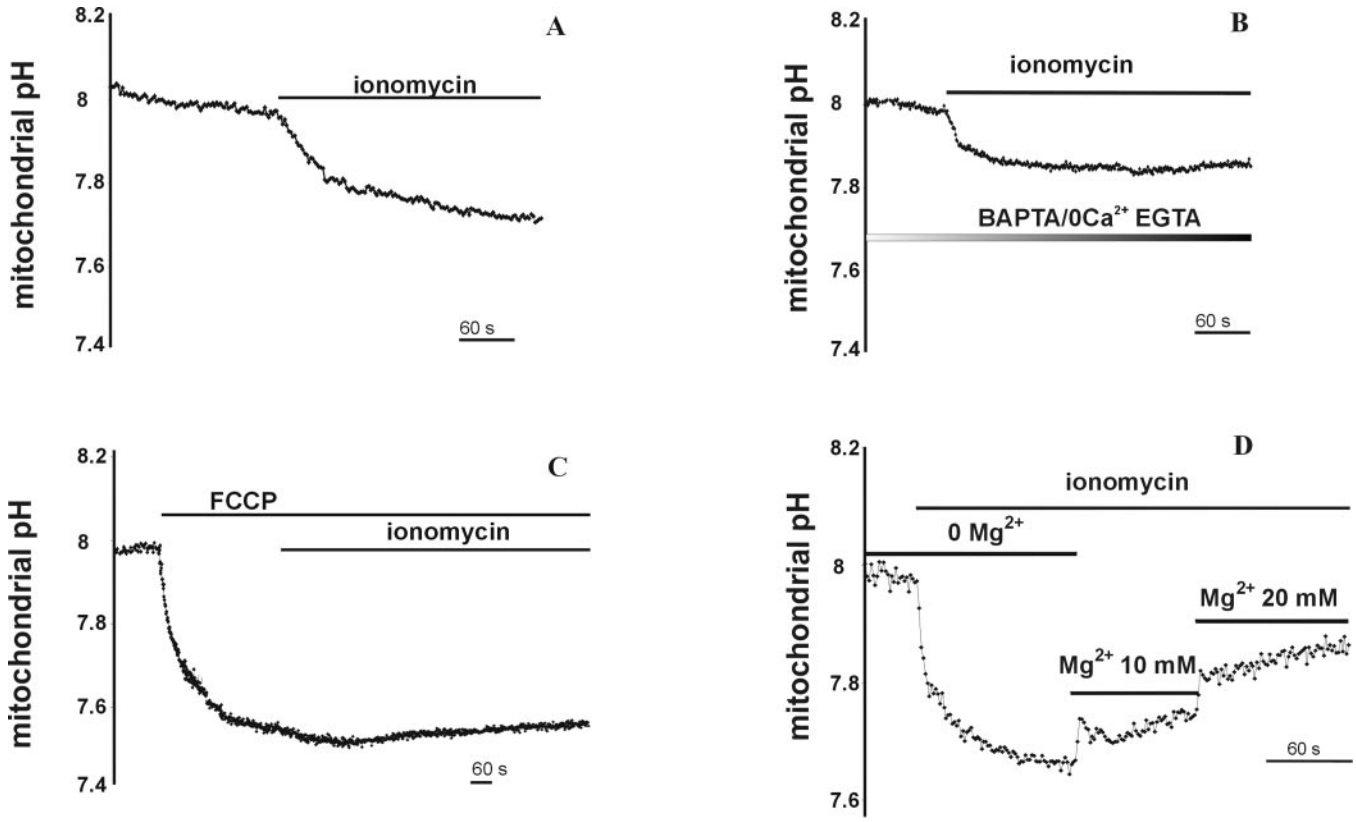


FIG. 9. **Calcium mobilization by ionomycin induces a drop in pH.** HeLa cells co-expressing mtAlpHi and mtECFP were perfused with ionomycin (*A*) or FCCP and ionomycin (*C*) for the indicated periods. In *A*, addition of ionomycin (1 $\mu$M) caused a marked acidification of the mitochondrial matrix pH. In *B* cells loaded with the permeant $Ca^{2+}$ chelator BAPTA (10 $\mu$M) in $Ca^{2+}$-free medium containing 1 mM EGTA were treated with ionomycin, and the typical drop on pH was observed. *C*, preincubation with FCCP (4 $\mu$M) prevented this effect. See "Results" for details. *D*, HeLa cells co-expressing mtAlpHi and mtECFP were first permeabilized (using 100 $\mu$M digitonin in a $Ca^{2+}$- and $Mg^{2+}$-free solution containing 1 mM ATP and 5 mM succinate) and then perfused with ionomycin (1 $\mu$M). In the continuous presence of ionomycin, the cells were perfused with $Mg^{2+}$ solutions at the indicated concentrations. A marked alkalinization of the mitochondrial matrix was observed upon addition of $Mg^{2+}$.

$Ca^{2+}$, such as that elicited by blocking the SERCA and allowing capacitative $Ca^{2+}$ influx, did not induce significant changes in matrix pH. Attempts to block phosphate movements (by treating the cells with *N*-ethylmaleimide; data not shown) were unsatisfactory because this drug has several nonspecific effects.

A significant alkalinization also occurs in the mitochondria of a subpopulation of neurons challenged with glutamate, a finding of utmost interest given that these doses of the neurotransmitter are known to cause massive cell death and that alkalinization of matrix pH has been suggested to be involved in activation of apoptosis (11, 12).

A rather unpredicted finding was, on the contrary, the acidification of matrix pH observed upon application of $Ca^{2+}$ ionophores such as ionomycin or A23187 to cells incubated in $Ca^{2+}$-free medium. This protocol is commonly employed to empty intracellular $Ca^{2+}$ stores and it is generally assumed that this is the only effect of the drugs under these conditions. mtAlpHi instead revealed that such a protocol results in an acidification of the matrix, similar to that caused by an uncoupler such as FCCP. Indeed, pretreatment of the cells with the uncoupler prevented the acidification induced by the $Ca^{2+}$ ionophores and similarly pretreatment with the ionophores abolished the acidification induced by the uncoupler.

The simplest interpretation of this finding would be the release from mitochondria of $Ca^{2+}$ in exchange of $H^+$. This explanation appears unlikely in view of the fact that (i) matrix $Ca^{2+}$ concentration in resting cells is very similar, if not identical to that of the cytoplasm; (ii) given the strong pH buffering power of the matrix, one would need a massive movement of $Ca^{2+}$ to elicit such a large pH change; ionomycin, on the contrary, is very inefficient at transporting $Ca^{2+}$ at nM concentrations. The demonstration that chelation of cytosolic and organelle $Ca^{2+}$ by loading the cells with BAPTA-AM in medium containing EGTA further supports this conclusion.

We have thus tested the possibility that the transport of another divalent cation in exchange of $H^+$ was responsible for such an acidification and we focused our attention on $Mg^{2+}$, the only cation with a free concentration in the matrix sufficiently high to justify such a large flux of $H^+$. Although we could not demonstrate directly the $Mg^{2+}$ movements, different experimental evidence supports such a conclusion, but only direct measurements of the cation concentrations within the matrix could finally prove the hypothesis.

In conclusion, pH changes occur in the matrix of mitochondria under a number of experimental conditions. While some of these changes were easily predicted (sodium acetate, $NH_4Cl$, uncouplers, olygomycin, or KCN), or expected corollaries of mitochondrial $Ca^{2+}$ uptake (histamine, glutamate), a few were highly unexpected, *i.e.* the acidification obtained with ionomycin and A23187. The changes in matrix pH here described should be carefully taken into consideration, not only because of their consequences for mitochondrial metabolism, but also because they can interfere with some of the widely used methodologies for measuring $Ca^{2+}$ handling by these organelles, primarily GFP-based $Ca^{2+}$ probes.

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# Mitochondrial pH Monitored by a New Engineered Green Fluorescent Protein Mutant

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