Mitochondria extrude protons across their inner membrane to generate the mitochondrial membrane potential ($\Delta \Psi_m$) and proton gradient ($\Delta p_{H_m}$) that both power ATP synthesis. Mitochondrial uptake and efflux of many ions and metabolites is driven exclusively by $\Delta p_{H_m}$, whose in situ regulation is poorly characterized. Here, we report the first dynamic measurements of $\Delta p_{H_m}$ in living cells, using a mitochondrial-targeted, pH-sensitive YFP (SypHer) combined with a cytosolic pH indicator (5,6-carboxy-SNARF). The resting matrix pH (~7.6) and $\Delta p_{H_m}$ (~0.45) of HeLa cells at 37°C were lower than previously reported. Unexpectedly, mitochondrial pH and $\Delta p_{H_m}$ decreased during cytosolic Ca$^{2+}$ elevations. The drop in matrix pH was due to cytosolic acid generated by plasma membrane Ca$^{2+}$ ATPases and transmitted to mitochondria by Pi/H$^+$ symport and K$^+$/H$^+$ exchange, whereas the decrease in $\Delta p_{H_m}$ reflected the low H$^+$-buffering power of mitochondria (~5mM, pH7.8) compared to the cytosol (~20mM, pH7.4). Upon agonist washout and restoration of cytosolic Ca$^{2+}$, mitochondria alkalinized and $\Delta p_{H_m}$ increased. In permeabilized cells, a decrease in bath pH from 7.4 to 7.2 rapidly decreased mitochondrial pH, whereas addition of 10 µM Ca$^{2+}$ caused a delayed and smaller alkalinization. These findings indicate that the mitochondrial matrix pH and $\Delta p_{H_m}$ are regulated by opposing Ca$^{2+}$-dependent processes of stimulated mitochondrial respiration and cytosolic acidification.

Mitochondria are mobile intracellular integrators of metabolic and ionic signalling. These multifunctional organelles generate ATP by oxidative phosphorylation, integrate signaling cascades leading to the release of pro-apoptotic factors (1), and shape cellular Ca$^{2+}$ signals by taking up and releasing Ca$^{2+}$ ions (2). By acting as transient Ca$^{2+}$ buffers, mitochondria alter the propagation of Ca$^{2+}$ waves, modulate the activity of plasma membrane channels and transporters (3,4), and facilitate the refilling of intracellular Ca$^{2+}$ stores (5,6), reviewed in (7). Mitochondrial metabolism and intracellular Ca$^{2+}$ signalling are closely linked. Increases in the free Ca$^{2+}$ concentration in the mitochondrial matrix ([Ca$^{2+}$]$_{mit}$) activates mitochondrial dehydrogenases to stimulate oxidative phosphorylation (8), and Ca$^{2+}$ elevations in the inter-membrane space stimulate the uptake of substrates of oxidative phosphorylation (9,10). Moreover, electrophoretic Ca$^{2+}$ uptake into the matrix is thought to reduce the electrical resistance for pumping protons out of the matrix (11).
requires a high concentration of protons (pH < 6.5) at the source P site of the enzyme (15), reviewed in (16). Efficient ATP synthesis thus not only requires a high driving force for protons, but also a low pH within the cristae of mitochondria. These data imply that a ΔpHm of > 1 pH unit must be maintained to enable the synthesis of ATP by respiring mitochondria.

The mitochondrial proton gradient (ΔpHm) is the sole driving force for the electroneutral transport of many ions and metabolites in and out of the mitochondrial matrix, while the activity of several mitochondrial ion exchangers is coupled, directly or indirectly, to the electroneutral movement of protons (17). For instance, the mitochondrial Na+ gradient is clamped close to ΔpHm by the electro-neutral mitochondrial 1Na+:1H+ exchanger (mNHE) [reviewed in (7)]. The sodium gradient, in turn, drives electrogenic mitochondrial 1Ca2+:3Na+ exchange (18) by the recently identified protein NCLX (19) that regulates mitochondrial Ca2+ levels. Electroneutral K+/H+ exchange catalyzed by the protein Letm1 is essential for mitochondrial ionic and volume homeostasis (20,21). The Letm1 protein was recently proposed to be an electrogenic Ca2+/H+ antiporter with a 1:1 stoichiometry (22), despite earlier studies indicating that Ca2+ enters mitochondrial as the fully charged species (23-25) and exits mitochondria with a 3H+:1Ca2+ stoichiometry (26). Regardless of whether Letm1 transports K+ or Ca2+ in exchange for H+, its exchange activity depends on ΔpHm. Finally, ΔpHm also influences the amplitude of [Ca2+]mitr elevations in that the mitochondrial Ca2+ buffering power is determined by the mitochondrial phosphate concentration, which is modulated by ΔpHm (27). Mitochondria therefore rely on ΔpHm to generate ATP, to move ions and metabolites, and to buffer Ca2+ ions.

The regulation of ΔμH+ has been extensively studied as it is essential for oxidative phosphorylation. Early approaches to estimate ΔμH+ in isolated mitochondria used potassium- and proton-selective electrodes or isotopes to detect changes in the external concentration of these ions in the presence of the K+ ionophore valinomycin to provide a diffusion potential (12,28). These experiments revealed that at low external K+ concentrations ΔpHm contributes up to 170 mV to the proton-motive force (12). In physiological conditions however (i.e. high K+ concentrations and no valinomycin) the situation is reversed and ΔΨm contributes most of the proton-motive force. These findings were validated by subsequent determinations of ΔΨm in isolated mitochondria and intact cells with fluorescent lipophilic cations [reviewed in (29)]. Our current knowledge of ΔpHm regulation is largely based on early experiments in isolated mitochondria that employed minimal sucrose buffers and H+/K+ ionophores such a nigericin (30,31). In intact cells, ΔpHm has been estimated around 1.0-1.2 pH units by isotopic measurements of weak acid or bases (32,33), thus contributing ~60 mV to ΔμH+. More recent studies using fluorescent indicators yielded ΔpHm values of 0.5-0.9 pH units based on static measurements separate measurements of matrix, cytosolic or inter-membrane space pH in sister cultures of live cells (34). These studies brought us closer to direct measurement of ΔpHm in live cells, but were unable to resolve the dynamic regulation of ΔpHm by factors like Ca2+ uptake, the activity of the mitochondrial permeability transition pore (35), and the presence of uncoupling proteins (36).

In this study, we simultaneously measured cytosolic pH using the well characterized dye SNARF and matrix pH using a ratiometric circularly permuted YFP. The concurrent measurements of pH in the cytosol (pHcyto) and mitochondrial matrix (pHmito) provided real-time measurement of the cytosol-matrix proton gradient, and thus of ΔpHm, in intact cells. Moreover, this approach enabled us to determine the proton buffering power of the matrix of intact mitochondria. Unexpectedly, we observed that [Ca2+]cyt elevations are associated with massive cytosolic and matrix pH decreases and consequent decreases in ΔpHm.

**EXPERIMENTAL PROCEDURES**

**Reagents:** Minimum essential medium with Glutamax (# 41090), fetal calf serum, penicillin/streptomycin, Lipofectamine 2000, 5-(and-6)-SNARF-1AM (C1272) and Fura-2 (F1201) were from Invitrogen. Histamine, 2-APB, orthovanadate, nigericin, monensin, antimycin, oligomycin and rotenone were from Sigma. Transfast was from Promega. The QuickChange II Site-Directed mutagenesis kit was from Stratagene.
Mutagenesis primers were from Microsynth (Switzerland). HyPer plasmids from Evrogen (Russia). YC3.6ypr and 4mitD3-CPV were kindly provided by Drs Amy Palmer and Roger Tsien.

**Cell culture and Transfection:** Culturing of HeLa cells was previously described (37). For epifluorescence microscopy, cells were plated on 25 mm glass coverslips in 35 mm culture dishes. For high throughput experiments, 5000-8000 cells/well were plated on µclear bottom, black-walled 96-well plates (# 655090, Greiner Bio One). Coverslips were transfected with 2 µg DNA and Lipofectamine 2000 (4 µl) or Transfast (3.5 µl) as per manufacturer’s instructions. Cells on 96-well plates were treated with 1.1 µl Lipofectamine 2000 and 0.4 µg DNA in 200 µl media per well for 6-24 hours and imaged two days later.

**Cell Permeabilization:** MitoSypHer expressing HeLa cells were permeabilized on the microscope with a 1 minute exposure to digitonin (100 µM) in Ca²⁺-free intracellular buffer: (in mM) 120 KCl, 10 NaCl, 1 H₃PO₄, 20 HEPES, 5 Succinic acid, 1 ATP-Mg²⁺, 0.02 ADP-K, 1MgCl₂ (1mM Mg²⁺ free); 0.5 EGTA adjusted to pH 7.4 with KOH. After digitonin washout, pH was stepped between 7.4 and 7.2 by turn-over of the bath solution. Addition of Ca²⁺ or drugs was performed by full bath turn-over. Total CaCl₂ and MgCl₂ was respectively 0.327 mM and 5.5 mM at pH 7.4, and 0.326mM - 5.43 mM at pH 7.2 to give ~10µM free Ca²⁺ buffered with 5mM HEDTA and 1mM ATP-Mg²⁺ as calculated with Max Chelator.

**Conversion of HyPer to SypHer:** In their description of HyPer, Belousov et al noted that mutation of either of two H₂O₂-sensing cysteine residues to a serine caused total loss of HyPer’s sensitivity to H₂O₂ (38). We mutated the first cysteine in cytosolic and mitochondria-targeted HyPer, to make SypHer. Briefly, the C199S mutation was performed with the QuickChange II site-directed mutagenesis kit as per manufacturer’s instructions using 12 reaction cycles with 5.5 min for elongation in each. Primers were used at 10-fold suggested concentration, Primers were 5' - agatgtcactttggegag - 3' (forward) and 5' - atgcggcaaggatgccatc - 3' (reverse) (Dr. Vsevolod Belousov, personal communication), with underlined nucleotides showing the position of the point mutation. PCR reactions were transformed into XL1-blue bacteria, and mini-preps (Gene Elute, Sigma) were prepared from clonal colonies for sequencing to confirm the C199S mutation and the absence of other mutations. Mutated plasmids were transfected into HeLa cells to confirm their sensitivity of pH and lack of response to H₂O₂ (200 µM).

**pH measurements:** Experiments were performed in HEPES-buffer (HBSS) solution containing (in mM): 140 NaCl, 5 KCl, 1 MgCl₂, 2 CaCl₂, 20 HEPES, 10 glucose, pH set to 7.4 with NaOH at 37°C. Ca²⁺-free solution contained 0.2 mM EGTA instead of CaCl₂. Cells grown on glass coverslips were inserted into a thermostatic chamber (Havard Apparatus, Holliston, MA) and solutions were changed by hand. Ratiometric pH and Ca²⁺ images were acquired with a 40x objective (1.3 NA, Zeiss Axiovert s100TV) and a cooled CCD camera (MicroMax, Roper Scientific, USA) (37). For pH imaging, SypHer (cytosolic or mitochondrial) was alternately excited for 200-300ms at 430 and 480 nm through a 505DCXR dichroic and imaged with a 535DF25 band-pass filters (Omega Optical, USA). For simultaneous mitochondrial/cytosolic pH measurements, SypHer expressing cells were loaded with SNARF (5µM, 0.2% DMSO, 0.01% pluronic F-127, 30 min, ambient temperature) followed by 20 min of de-esterification before 10 min of equilibration on the heated stage. SypHer was imaged as above, and SNARF was excited at 480 nm for 200ms through same dichroic and imaged with 580DF30 and 640DF35 filters (Omega Optical). Image pairs and quartets were typically acquired every 2-5 seconds. Fluorescence ratios (F480/430 SypHer, F640/580 SNARF) were calculated in MetaFluor 6.3 (Universal Imaging) and analyzed in Excel (Microsoft, USA) and GraphPad Prism 5.01 (GraphPad, CA, USA). For each cell a 6-point calibration curve was fitted to a variable slope sigmoid equation with 1/Y weighting and constraining the top of the curve to 30 (GraphPad Prism 5.01).

**Ca²⁺ imaging:** Cytosolic Ca²⁺ was measured with fura-2 (2µM) or YC3.6 (39), and mitochondrial Ca²⁺ with 4mitD3-CPV on the epifluorescence
system described above. Fura-2 was excited alternately at 340 and 380 nm (200-300 ms/λ) through a 430DCLP dichroic and 510WB40 emission filter when recording only Fura-2 or through a 505DCXR dichroic and 535DF25 filter when measured simultaneously with SypHer. Cameleons were excited at 430 nm through a 455DRLP dichroic and alternately imaged with 475DF15 and 535DF25 emission filters (200-400 ms/λ, Omega Optical).

**Cell lysis, mitochondrial isolation, Western blotting:** Whole cells were lysed in a glass homogenizer. The lysate was centrifuged at 14,000 x g for 20 min and the protein content of the supernatant was determined using a BCA Protein Assay (Pierce). Mitochondrial fraction was obtained by differential centrifugation as previously reported (40). 20 µg of total protein (from cytosolic or mitochondria fraction) was loaded per lane of SDS-PAGE. For immunoblotting, proteins were transferred onto nitrocellulose membrane and probed with the following antibodies from Santa Cruz Biotechnology: anti- GFP (sc-9996), anti-Tom20 (sc-11415), and anti- α-tubulin (T9026) from Sigma. Horseradish peroxidise-conjugated Secondary antibodies (Amersham) were used, followed by detection by chemiluminescence (Amersham).

**Immunocytochemistry & Confocal Imaging:** 48h after transfection cells were fixed using 4% paraformaldehyde for 15min, permeabilized with 0.1% Triton X-100 for 10min and incubated in blocking buffer IgG 2µg/ml for 1h. Fixed cells were probed with rabbit polyclonal anti-Tom20 (1/100) for 10h at 6°C and labelled with Alexa Fluor 568 conjugated goat anti-rabbit IgG (1/1000) from Sigma. Confocal images were acquired on a Leica SP5 2 photon microscope using a 63X oil immersion objective. MitoSypher was exited at 488nm and fluorescence emitted between 510-540nm was collected. Alexa-fluor 568 was exited at 561nm and fluorescence emitted between 600-630nm was collected. The confocal pinhole was 154 µm (1 airy disk) for SypHer and 204µm (1.25 airy disk) for Tom20.

**Live-cell Confocal Imaging:** Confocal images were acquired with a Zeiss LSM510meta, using a 63x Achromplan water immersion objective (NA 0.95) and 12-bit acquisition mode with zoom set to give 130 nm pixels. For mitoSypher / MitoTracker dual labelling, two days after mitoSypher transfection cells were incubated with 25 nM MitoTracker Red CMXRos for 45min at 37°C in normal culture media. Media was replaced with HBSS (ambient temperature) immediately before image acquisition. Images were acquired with simultaneous 488/561 nm excitation with sequential 490nm and 565 nm dichroics and 505-550 bandpass emission (SypHer) and 575 longpass (MitoTracker Red CMXRos).

**Calculating of $H^+$ buffering power and fluxes:** Cytosolic and mitochondrial $H^+$ buffering powers ($\beta$) were calculated by measuring pH changes in cells exposed to permeant weak acid (butyric acid, BTA, pKa 4.82) or weak base (trimethylamine, TMA, pKa 9.80) and estimating the change in internal acid/base concentration as previously described (41). Some investigators assume that weak acids (bases) fully deprotonate (protonate) at physiological pH to simplify calculations. We found that this assumption systematically over-estimated the calculated changes in weak acid and base, so we relied on exact calculation by the Henderson-Hasselbach equation. To ensure that we measured intrinsic cytosolic and matrix buffering power, the Na’/H’-exchanger was inhibited with amiloride (100µM) and the respiratory chain was inhibited with rotenone (5µM), antimycin (5µM) and oligomycin (5µg/ml). The concentration of TMA in compartment j (cytosol or mitochondria) was estimated by:

$$[\text{TMAH}^+]_j = \frac{[\text{TMAH}^+ + \text{TMA}^+]}{1+10^{(pK_a-pH_j)}} 	imes 10^{(pK_a-pH_j)}$$

where compartment k (extracellular space or cytosol) is the compartment immediately external to j and $pK_a^{\text{TMA}}$ is assumed to be equal in all compartments. For BTA, the same equation is used, but $(pH_j - pK_a)$ replaces $(pK_a - pH_j)$. Subsequently $\beta$ is calculated by:

$$\beta_j = \frac{\Delta[\text{TMAH}^+]}{\Delta pH_j}$$

(calculated before and after a change in [TMA]bath and $\Delta pH_j$ is measured as the mid-point pH between changes in [TMA]bath. Simultaneous measurements of pHcyto and pHmito permitted $\beta_{\text{mito}}$ to be calculated from eq.1 where the cytosol is compartment k.)
High throughput pH imaging: Separate halves of a 96-well plate were transfected with SypHer and mitoSypHer. 48hr later, culture media was replaced with HBSS and the plate was placed in an ImageXpress Micro plate reading microscope system (Universal Imaging, Sunnyvale, California, USA) (humidified, 37°C). Using laser-assisted auto-focusing and automated image acquisition (20x objective, 0.5 NA) ratio images (F480: ex1 470/45 nm, em1 520/40; F430: ex2 435/25 nm, 465 nm dichroic2, em2 480/35 nm) were acquired over the same grid pattern in each well (4-12 sites/well) with a 12-bit CCD camera. For histamine stimulations, 100 µl of 2x-concentrated histamine was added to 100µl HBSS in each well with a multi-channel pipette and recording was started within 1 minute of addition. On each plate SypHer and mitoSypHer ratios were calibrated such that intracellular pH for one well for each probe was clamped at 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 (Fig. S3A). We created automated algorithms (MetaXpress, Universal Imaging) to define regions around cells in F480 images, to calculate background fluorescence and subtract it from the average F80 and F430 fluorescence intensity in each defined cell region. Single-cell fluorescence values (50 – 400 per well) were transferred to Excel for sorting and calibration of ratio values. For each well, cells with ratio values greater than two standard deviations from the mean value were excluded, and individual ratio values were calibrated against a standard curve generated on each plate.

RESULTS

Probe generation and validation: While testing the properties of HyPer, a commercial H₂O₂-sensing probe based on a circularly permuted YFP (38), we observed that HyPer exhibited ratiometric responses to NH₄⁺-induced (30 mM) matrix alkalinization of similar amplitude to its responses to H₂O₂ (200 µM) (Fig. 1Aii). We reasoned that HyPer could provide the basis for a ratiometric pH-sensor with excellent mitochondrial targeting and brightness. We generated a pH-specific version of HyPer that we dubbed SypHer (Synthetic pH sensor) by mutating one of HyPer’s two H₂O₂-sensing cysteine residues (C199S) as described by Belousov et al. (38). As expected, cells expressing cytosolic SypHer still exhibited large fluorescence changes upon addition of NH₄⁺, but failed to respond to H₂O₂ (Fig 1Aii). We then characterized the properties of the SypHer probe in vitro, using protein extracts from HeLa cells expressing cytosolic SypHer. As shown in Fig. S1, the fluorescence intensity increased markedly with pH at excitation wavelength exceeding 435 nm, with a peak around 490 nm. The 490/420 fluorescence ratio was not altered by the addition of millimolar concentrations of Ca²⁺, PO₄⁻, and H₂O₂ (Table 1) but increased by ~5 fold when the bath pH was increased from 7.0 to 7.8 (Fig. S1B). MitoSypHer was efficiently targeted to the mitochondrial matrix by tandem mitochondrial localization sequences, as demonstrated by its colocalization with the mitochondrial resident protein TOM20 and with MitoTracker (Fig. 1B, Pearson coefficients 0.88±0.03 and 0.91±0.06, n= 4 and 5 cells, respectively) and enriched reactivity for an anti-GFP antibody in mitochondrial fractions (Fig. S2A). Mitochondria-targeted SypHer (mitoSypHer) had spectral properties similar to its parental probe (Fig. 1C), and displayed opposite changes in fluorescence at λex=420 and λex=490 when the pH of the organelle was varied with H⁺ ionophores (Fig. S2B). Using an automated microscope to image cells on 96-well plates, equilibration of the extracellular and organelar pH with H⁺ ionophores confirmed that the mitoSypHer ratio reported variations in local pH and that resting pHmito in most cells was between 7.5 - 8.0 (Fig. S3A). pH titration curves generated from different wells of the 96-well plate clamped at specific pH values and with cells expressing cytosolic or mitochondria-targeted SypHer closely overlapped (Fig. S3B), demonstrating that SypHer’s pH responses were not altered by the matrix environment and that the calibration protocol equilibrated both compartments. In situ calibration of mitoSypHer ratio against pH showed a pKa of 8.71±0.05 and a Hill slope of 0.96±0.05 (Fig. 1D) with an impressive 20-fold increase in ratio when pH increased from 7 to 10. The ratio increased 4-fold in the more physiological pH range of 7 to 8, (Fig. 1D, inset), permitting accurate measurements of pHmito. Having validated the probe, we next measured resting pHmito on the standard epifluorescence microscope, generating an autonomous calibration curve for each cell imaged. To our surprise, resting pHmito (7.61 ± 0.02, min
7.26, max 7.91, 79 cells, Fig. 1E) was notably lower than the values of 7.8-8.1 previously reported in intact cells (42-44). To confirm these observations we measured pH_mito and pH_cyto with SypHer in a large number of cells, by imaging 96-well plates (Fig. S3). Mitochondrial pH was 7.64 ± 0.14 (mean ± SD) at 37°C and significantly higher (7.77 ± 0.26, mean ± SD) at 25°C (Fig. 1E), consistent with reports that isolated mitochondria are more polarized at 28°C vs. 37°C (45). In contrast, pH_cyto was slightly higher at 37°C (7.19 ± 0.14) than at 25°C (7.12 ± 0.14). Consequently, the pH gradient across the inner mitochondrial membrane, \( \Delta \text{pH}_m \), averaged 0.46 unit s in resting, non-stimulated HeLa cells at 37°C and increased to 0.65 units when the temperature was decreased to 25°C. The resting pH_mito and \( \Delta \text{pH}_m \) of quiescent cells at physiological temperatures were thus lower than previously reported.

Mitochondrial pH decreases during cytosolic Ca\(^{2+}\) elevations. Cytosolic Ca\(^{2+}\) elevations boost mitochondrial metabolism, but whether this effect involves net changes in pH_mito is controversial because both alkalisation and acidification of mitochondria have been reported during [Ca\(^{2+}\)]_cyt elevations (42,43,46-48). To determine how changes in [Ca\(^{2+}\)]_cyt alter \( \Delta \text{pH}_m \), HeLa cells expressing mitoSypHer were loaded with the Ca\(^{2+}\)-sensitive dye fura-2 to monitor [Ca\(^{2+}\)]_cyt and pH_mito simultaneously. The [Ca\(^{2+}\)]_cyt elevations evoked by the Ca\(^{2+}\)-mobilizing agonist histamine were associated with a significant decrease in pH_mito (Fig. 2A), but the [Ca\(^{2+}\)]_cyt and pH_mito responses had different temporal profiles. The [Ca\(^{2+}\)]_cyt elevation exhibited a transient peak followed by a sustained plateau, whereas the pH_mito decay was monophasic (Fig. 2A), even in cells that exhibited Ca\(^{2+}\) oscillations during the sustained phase of the Ca\(^{2+}\) signal (Fig. 2A, insets). Parallel pH_cyto recordings with cytosolic SypHer revealed parallel decreases in pH_cyto during [Ca\(^{2+}\)]_cyt elevations, but with a smaller amplitude than decreases of pH_mito (Fig. 2B). Interestingly, the larger decrease in pH_mito than pH_cyto was more pronounced during the second histamine stimulation (Fig. 2B). Cell-to-cell variability limited our ability to measure precisely \( \Delta \text{pH}_m \) in individual cells from independent recordings of pH_cyto and pH_mito. To overcome this limitation we simultaneously measured pH_mito using mitoSypHer and pH_cyto using the pH indicator SNARF, a red-shifted dye whose fluorescence emission does not overlap with the yellow fluorescence of mitoSypHer (Fig. 3B). Calculating the cell-wise difference between the pH_mito and pH_cyto recordings (black and blue traces, respectively), these concurrent measurements enabled us to follow \( \Delta \text{pH}_m \) (red trace on bottom panel) in intact cells. As shown in Fig. 3A, each histamine stimulation decreased \( \Delta \text{pH}_m \) by ~0.05 (range: 0.06 to -0.28, Fig. 3Ci) as drops in pH_mito typically exceeded those in the cytosol. Upon histamine removal, pH_cyto recovered to resting levels, while a pH_mito overshoot was typical following successive stimulations (Fig. 3A). As a result, \( \Delta \text{pH}_m \) increased from 0.15 ± 0.02 in “resting” cells to 0.44 ± 0.02 following recovery from a third histamine stimulation (Fig. 3Ci). Using the high throughput imaging, we also observed that histamine-mediated acidification was concentration dependent and maintained during 25-30 minutes of stimulation with only modest recovery (Fig. S3D).

[Ca\(^{2+}\)]_cyt-dependency of the decreases in pH_mito and pH_cyto. A close inspection of Ca\(^{2+}\) and pH responses evoked by histamine revealed that the kinetics of acidification closely matched the instantaneous integral of the Ca\(^{2+}\) elevation, suggesting that acid accumulation was proportional to the total cytosolic Ca\(^{2+}\) load rather than its instantaneous concentration (Fig. 4A). The typical peak-and-plateau [Ca\(^{2+}\)]_cyt response to histamine is due to initial Ca\(^{2+}\) release from the endoplasmic reticulum (ER) through inositol(1,4,5)-trisphosphate receptors (InsP\(_3\)R) followed by sustained Ca\(^{2+}\) influx across store-operated Ca\(^{2+}\) entry (SOCE) channels. To determine if [Ca\(^{2+}\)]_cyt was necessary for the intracellular acidification, we treated cells with 2-APB, an inhibitor of both InsP\(_3\)R intracellular Ca\(^{2+}\) release channels and store-operated Ca\(^{2+}\) channels. As expected, 2-APB (75µM) inhibited both the release and influx components of [Ca\(^{2+}\)]_cyt elevations measured with the fret-based YC3.6, reducing the integrated Ca\(^{2+}\) response by 86.2 ± 1.7% (Fig. S4A&C). In parallel, 2-APB impaired pH_cyto and pH_mito decreases and completely prevented the loss of \( \Delta \text{pH}_m \) evoked by histamine (Fig. S4B,D & E). To separate the influx from the release component of the [Ca\(^{2+}\)]_cyt elevation, cells were treated with histamine in the presence of...
thapsigargin (1 µM) to inhibit the sarco/endoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA) and to deplete ER Ca\(^{2+}\) stores. Subsequent restitutions of external Ca\(^{2+}\) caused large \([Ca^{2+}]_{cyt}\) elevations due to Ca\(^{2+}\) entry through SOCE channels that were sufficient to induce massive decreases in pH\(_{cyto}\) and pH\(_{mito}\) (Fig. 4Bi&ii). Rapid Ca\(^{2+}\) extrusion was initiated immediately upon removal of extracellular Ca\(^{2+}\), whereas the onset of pH recovery was delayed until \([Ca^{2+}]_{cyt}\) was almost at resting levels (Fig. 4Ci&ii, insets). Thus Ca\(^{2+}\)-dependent acidification did not require the activity of SERCA, and all conditions that caused \([Ca^{2+}]_{cyt}\) elevations decreased both pH\(_{cyto}\) and pH\(_{mito}\).

**PMCA underlies the Ca\(^{2+}\)-dependent decreases in pH.** Like SERCA, PMCA catalyzes H\(^{+}\)/Ca\(^{2+}\) counter-transport (49,50), reviewed in (51,52). Several lines of evidence indicate that the activity of the PMCA could account for the drop in pH associated with \([Ca^{2+}]_{cyt}\) elevations. First, La\(^{3+}\), at millimolar concentrations known to inhibit the PMCA (53), completely inhibited histamine-induced decreases in pH\(_{cyto}\) (not shown) and pH\(_{mito}\), while enhancing \([Ca^{2+}]_{cyt}\) elevations (Fig. 5A). Since La\(^{3+}\) is not a specific inhibitor of PMCA, we attempted to isolate the activity of the PMCA by treating cells with thapsigargin and reducing extracellular Na\(^{+}\) to 0-5 mM to inhibit the NCX. Under these conditions, the activity of the PMCA is directly reflected by the rapid decrease in \([Ca^{2+}]_{cyt}\) upon removal of extracellular Ca\(^{2+}\). As expected, La\(^{3+}\) (5 mM) rapidly and reversibly blocked this \([Ca^{2+}]_{cyt}\) recovery (Fig. 5Bi), with an apparent IC\(_{50}\) of 0.2-0.3 mM (data not shown). Importantly, La\(^{3+}\) not only completely blocked the pH\(_{cyto}\) decrease initiated by \([Ca^{2+}]_{cyt}\) elevation (not shown) but it revealed a Ca\(^{2+}\)-dependent pH\(_{mito}\) alkalization, particularly under conditions of low extracellular Na\(^{+}\) (Fig. 5Bii). The PMCA is also inhibited at alkaline extracellular pH (pH\(_{o}\)) as the ATPase is starved for protons to exchange with cytosolic Ca\(^{2+}\) (54). Increasing pH\(_{o}\) to 8.2 - 8.8 prevented the rapid recovery of \([Ca^{2+}]_{cyt}\) upon removal of histamine, while subsequent restoration of physiological pH\(_{o}\) (7.4) caused an immediate decrease in \([Ca^{2+}]_{cyt}\) demonstrating the inhibition of the PMCA at alkaline pH\(_{o}\) (Fig. 5Ci). In parallel experiments, alkaline pH\(_{o}\) prevented the histamine-induced drop in pH\(_{cyto}\) and pH\(_{mito}\) (Fig. 5Cii), consistent with a role for the PMCA in the acid generation. Inhibition of PMCA has also been shown for orthovanadate, with sensitivities ranging from micromolar to millimolar concentrations (53). We observed little to no inhibition of histamine-induced acidification at micromolar concentrations of orthovanadate, but 1 and 10 mM orthovanadate inhibited both pH\(_{cyto}\) and pH\(_{mito}\) acidifications by ~30% (data not shown). Thus, regardless of the approach used to inhibit PMCA, decreased Ca\(^{2+}\) extrusion was consistently associated with decreased cellular acidification, strongly suggesting that PMCA was the main source of the acid generated during \([Ca^{2+}]_{cyt}\) elevations.

**Influence of mitochondrial H\(^{+}\)-buffering power on \(\Delta pH_m\).** Mitochondria consistently showed larger decreases in pH than concomitant changes in the cytosol during histamine stimulations. The larger pH\(_{mito}\) decrease caused a net loss of \(\Delta pH_m\) during each agonist application (Fig. 3), to the extent that the loss of \(\Delta pH_m\) showed a linear correlation with the absolute value of pH\(_{mito}\) immediately prior to histamine addition (not shown). The net loss of \(\Delta pH_m\) during histamine-mediated pH\(_{cyto}\) acidification could reflect the facilitated import of protons across the inner mitochondrial membrane. Alternatively, the different amplitudes of decreases in pH\(_{mito}\) and pH\(_{cyto}\) might reflect different proton buffering capacities (\(\beta\)) of the cytosol and mitochondria. High concentrations of cytosolic pH buffers mitigate intracellular pH changes during surges of intracellular acid production. A lesser pH buffering capacity of mitochondria compared to the cytosol might therefore cause a greater decrease in pH\(_{mito}\) than pH\(_{cyto}\) for an identical acid load. The pH buffering capacity of the cytosol (\(\beta_{cyto}\)) is reported to be 20-30 mM (55), but the intrinsic buffering power of mitochondria (\(\beta_{mito}\)) has not been determined to date. To obtain this parameter, we simultaneously measured the pH\(_{mito}\) and pH\(_{cyto}\) changes evoked by the addition of varying concentrations of permeant weak acids (butyric acid, BTA) or bases (trimethylamine, TMA), the former illustrated in Fig. 6Ai. This well-established procedure (41) enabled us to determine the buffering power of the cytosol and of mitochondria, over the physiological range of pH 6.8-8.4. As shown in Fig. 6Aii, the pH dependency of \(\beta_{cyto}\) and \(\beta_{mito}\) was similar (pK\(_A_{cyto}\)
of the amplitude of the drop in pH mito (compartment. Combined, these findings show that the buffering capacity of mitochondria rapidly decreases as the organelle alkalinizes. This strongly suggests that the reduced H\(^+\) buffering capacity of mitochondria at alkaline pH underlie the loss of \(\Delta p\text{H}_m\) during histamine stimulation.

To quantify the effect of the differential buffering capacity of the two compartments, we used the measured \(\beta_{\text{cyto}}\) and \(\beta_{\text{mito}}\) values to calculate the net fluxes of protons (jH\(^+\)) underlying the typical pH\(\text{cyto}\) and pH\(\text{mito}\) responses in a random subset of cells (Fig. 6B). As expected, correlation of the amplitude of the drop in pH\(\text{mito}\) (\(\delta\text{pH}_{\text{mito}}\)) with the drop in pH\(\text{cyto}\) (\(\delta\text{pH}_{\text{cyto}}\)) produced a regression slope greater than one, consistent with the loss of \(\Delta p\text{H}_m\) (Fig. 6Ci). When changes in pH were converted to net H\(^+\) fluxes (jH\(^+\)) with the formula: jH\(^+\) = \(\delta\text{pH}_i \times \beta_i\), the positive correlation was retained but with a slope significantly less than unity (Fig. 6Cii). This meant that the flux of protons reaching the mitochondrial matrix was smaller than the flux of protons entering the cytosol, as expected given that the inner mitochondrial membrane is not freely permeable to protons. Thus the loss of \(\Delta p\text{H}_m\) was likely due to the difference between \(\beta_{\text{mito}}\) and \(\beta_{\text{cyto}}\) at physiological pH values in each compartment. Combined, these findings show that the absolute value of pH\(\text{mito}\) prior to a stimulus like histamine and the pH-dependence of \(\beta_{\text{mito}}\) are important, previously unrecognized determinants of the changes in pH\(\text{mito}\) and \(\Delta p\text{H}_m\) occurring in living cells.

\textit{Ca}^{2+} and H\(^+\) have opposite effects on mitochondrial matrix pH. The drop in pH\(\text{mito}\) observed during \([\text{Ca}^{2+}]_{\text{cyt}}\) elevations is at odds with the known effect of mitochondrial Ca\(^{2+}\) uptake to cause matrix alkalinisation (17). To study whether the decrease in mitochondrial pH was due to the concomitant cytosolic acidification that occurred during the \([\text{Ca}^{2+}]_{\text{cyt}}\) elevation, we measured the changes in pH\(\text{mito}\) in permeabilized cells exposed to Ca\(^{2+}\) or pH changes. As shown in Fig. 7, a transient decrease in bath pH from 7.4 to 7.2 rapidly and reversibly decreased the matrix pH by ~0.15 pH units. Subsequent addition of 10 \(\mu\text{M Ca}^{2+}\) at pH 7.4 evoked a matrix alkalinisation that developed more slowly and that was of smaller amplitude, increasing matrix pH by ~0.08 pH unit. Parallel experiments in cells expressing 4mitD3-CPV revealed that addition of Ca\(^{2+}\) to permeabilized cells caused robust \([\text{Ca}^{2+}]_{\text{mit}}\) elevations that were largely prevented by inhibition of the Ca\(^{2+}\) uniporter with Ru360 (Fig. S5A). We then added 10 \(\mu\text{M Ca}^{2+}\) during the switch to pH 7.2 to mimic the Ca\(^{2+}\) and pH changes occurring in intact cells stimulated with histamine. This protocol caused a rapid decrease in pH\(\text{mito}\) followed by a slow recovery to a steady-state value ~0.09 pH units more acidic than the initial pH\(\text{mito}\) (Fig. 7). Thus mitochondria showed the expected pH\(\text{mito}\) increase in response to Ca\(^{2+}\) elevations, but this alkalinization was masked by a larger acidification when pH\(\text{cyto}\) was decreased concomitantly.

\textit{Contributions of mitochondrial transporters to mitochondrial H\(^+\) fluxes.} The large flux of protons reaching the mitochondrial matrix when the cytosol acidifies indicates that pH\(\text{cyto}\) and pH\(\text{mito}\) are in dynamic equilibrium. This is not surprising, given the multiplicity of H\(^+\)-coupled transporters present in the inner mitochondrial membrane, and we then attempted to identify pharmacologically the transporters involved. Cells were stimulated repeatedly with histamine as in Fig. 3 and inhibitors were added 5 minutes before the third stimulation. We first inhibited the electron-transport chain (ETC) with rotenone and antimycin to block complexes I and III, respectively. As expected, inhibition of the ETC caused an immediate decrease in pH\(\text{mito}\) and collapsed \(\Delta p\text{H}_m\) to 30% of resting levels. In these conditions, the decrease in pH\(\text{mito}\) evoked by histamine closely matched that of pH\(\text{cyto}\), and the histamine-induced loss of \(\Delta p\text{H}_m\) was precluded (Fig 8A). To quantify the effect of ETC inhibition on mitochondrial H\(^+\) fluxes, the ratio of net jH\(^+\)\(\text{mito}\) to net jH\(^+\)\(\text{cyto}\) evoked by histamine was compared in single cells before and after the addition of inhibitors (or vehicle) to
account for cell to cell variability in $j_{H}^{+}_{cyt}$. As shown in Fig. 8C, ETC inhibition reduced $j_{H}^{+}_{mito}$: $j_{H}^{+}_{cyt}$ by $\sim 28 \pm 18\%$. We then used oligomycin to inhibit the F$_{1}$F$_{0}$-ATP synthase. Contrary to our expectations, the addition of oligomycin did not increase pH$_{m}$ over a 5 minute period in a majority of cells, possibly because mitochondria were already maximally alkaline after two successive stimulations with histamine. Oligomycin had no apparent effect on the subsequent decrease in pH$_{cyt}$, pH$_{mito}$ and $\Delta$H$_{m}$ evoked by a third addition of histamine, and calculation of the protons fluxes revealed that oligomycin did not significantly reduced the average $j_{H}^{+}_{mito}$:$j_{H}^{+}_{cyt}$ (Fig. 8C).

Bongkrekic acid, an inhibitor of the adenine nucleotide translocase (ANT), rapidly and selectively acidified mitochondria but did not significantly alter the $j_{H}^{+}_{mito}$:$j_{H}^{+}_{cyt}$ (Fig. 8C). Thus, only inhibition of the ETC consistently decreased proton fluxes (by 20-25%), but this inhibition was associated with a decreased proton-motive force. Next, we investigated whether the mitochondrial Na$^{+}$/Ca$^{2+}$-exchanger (NCLX), the major mitochondrial Ca$^{2+}$ extruder of HeLa cells (56), participates in the rapid flux of protons into the matrix during cytosolic Ca$^{2+}$ elevations. NCLX activity requires a mitochondrial Na$^{+}$ gradient generated and maintained by the mNHE, thus effectively coupling the extrusion of one Ca$^{2+}$ ion with the entry of three protons into the mitochondrial matrix (7). To examine the contribution of the mNCX/mNHE axis to net mitochondrial H$^{+}$ flux, we used CGP-37157 to inhibit the NCLX (Fig. 8A). CGP-37157 reduced and delayed the onset of pH$_{cyt}$ and pH$_{mito}$ acidification, consistent with Ca$^{2+}$ sequestration in mitochondria and reduced PMCA activity. Importantly, CGP-37157 reduced $j_{H}^{+}_{mito}$:$j_{H}^{+}_{cyt}$ by $\sim 45\%$ (Fig. 8C), suggesting that the mNCX/mNHE axis is implicated in the rapid transmission of protons from the cytosol to the mitochondrial matrix.

As our pharmacological analysis of intact cells did not reveal a single clear mechanism of rapid proton flux across the mitochondrial membrane, we turned to permeabilized cells to clarify the mechanisms contributing to the high permeability of mitochondria for protons. As shown in Fig. 8B, the combination of oligomycin, antimycin, and rotenone rapidly decreased pH$_{mito}$, consistent with inhibition of the ETC. Under these conditions, the amplitude of the drop in pH$_{mito}$ evoked by a rapid drop in bath pH was reduced by $\sim 38\%$ and the calculated proton flux by 30% (Fig. 8D). In contrast, resting pH$_{mito}$ as well as acid-induced proton fluxes were not affected by Ru360 and CGP 37157 (Fig. 8B&D). Consistent with inhibition of mitochondrial Ca$^{2+}$ uptake and extrusion, Ru360 reduced the pH$_{mito}$ elevations evoked by the addition of Ca$^{2+}$ to permeabilized cells (Fig. S5B) whereas CGP 37157 potentiated the Ca$^{2+}$-induced matrix alkalinization (Fig. S5C). Finally, we tested the effects of the Pi/H$^{+}$ symport inhibitor mersalyl. Mersalyl (100 µM) induced a rapid increase in matrix pH followed by a subsequent decrease that was associated with a 70% increase in proton flux (Fig. S6A). The initial alkalinization upon mersalyl addition is consistent with inhibition of coupled Pi/H$^{+}$ entry, but the subsequent drop in matrix pH and increased proton flux suggest that the inner membrane has been damaged by mersalyl. We then turned to ion substitution experiments to identify the H$^{+}$ transporters involved in the rapid flux of protons across the inner mitochondrial membrane. As shown in Fig. S6B, phosphate removal increased pH$_{mito}$ whereas phosphate addition decreased pH$_{mito}$, consistent with coupled Pi/H$^{+}$ transport. Conversely, potassium removal (120 mM KCl substituted with 235 mM sucrose) decreased pH$_{mito}$ whereas subsequent addition of 50 mM KCl increased pH$_{mito}$ (Fig. S6C). Both effects are consistent with K$^{+}$/H$^{+}$ exchange, since in our short-term recordings mitochondria are not depleted of K$. A drop in pH$_{mito}$ was also observed in sucrose medium when the bath pH was switched from 7.4 to 7.2 (data not shown). The drop in pH$_{mito}$ could reflect increased K$^{+}$/H$^{+}$ exchange activity (i.e. more K$^{+}$ leaving the matrix in exchange for additional external protons). These recordings show that changes in the cytosolic concentration of Pi and K$^{+}$ alter the matrix pH in the direction predicted by Pi/H$^{+}$ symport and K$^{+}$/H$^{+}$ exchange.

Together, the pharmacological and ion substitution experiments indicate that the Ca$^{2+}$ uniporter and the Na$^{+}$/Ca$^{2+}$ exchanger NCLX do not substantially facilitate the flux of protons into mitochondria during changes in environmental pH. Instead, Pi/H$^{+}$ symport and K$^{+}$/H$^{+}$ exchange activity likely contributes to the rapid adaptation of the mitochondrial matrix pH to changes in cytosolic pH.
DISCUSSION

In this study, we report the first time-resolved quantitative measurements of the changes in the mitochondrial pH gradient ($\Delta p_{\text{H}_m}$) and of the trans-mitochondrial proton fluxes occurring in single living cells during physiological stimulation. The determination of $\Delta p_{\text{H}_m}$ was permitted by concurrent recordings of mitochondrial and cytosolic pH by combining our new mitochondria-targeted, ratiometric indicator SypHer with the cytosolic dye SNARF-1. These measurements revealed several novel and unexpected aspects of mitochondrial ionic homeostasis.

First, the resting pH of mitochondria in HeLa cells was unexpectedly low, averaging 7.8 at 25°C and only 7.6 at 37°C, a value that increased to 7.8 upon repetitive stimulation with histamine. Previous studies using pH-sensitive GFP mutants reported higher resting pH$_{\text{mito}}$ values of 7.8–8.2 in HEK-293, Jurkat and HeLa cells (42,44,57). Others however reported lower pH$_{\text{mito}}$ values, similar to those we report here. A resting pH$_{\text{mito}}$ of 7.8 was reported in ECV304 endothelial cells using a mitochondrial EYFP (34) and a pH$_{\text{mito}}$ of 7.7 in MDCK cells with the ratiometric dye SNARF-1 (58). Recently, we reported a resting pH$_{\text{mito}}$ of 7.2 in rat pancreatic $\beta$ cells that increase to 7.7 upon nutrient stimulation (46). pH$_{\text{mito}}$ is an important parameter that determines the ability of mitochondria to fulfill their metabolic and homeostatic functions. pH$_{\text{mito}}$ increases early during apoptosis (44), and a low pH$_{\text{mito}}$ modulates the opening of the permeability transition pore (59,60), reviewed in (61). In pancreatic $\beta$ cells, an alkaline pH$_{\text{mito}}$ is required for the delayed $[\text{Ca}^{2+}]_{\text{cyto}}$ that sustains the secretion of insulin in response to glucose (46). Our current measurements with SypHer indicate that the commonly assumed resting pH$_{\text{mito}}$ of 7.8 likely overestimates typical resting pH$_{\text{mito}}$ in HeLa cells and several other cell types. Despite this lower than expected value for pH$_{\text{mito}}$, our $\Delta p_{\text{H}_m}$ values of 0.5–0.7 pH units derived from static high-throughput measurements and 0.2–0.4 pH units derived from dynamic single-cell imaging agree well with the values of 0.3–0.8 pH units reported in earlier studies with GFP-based probes (34,43). Since the inter-membrane space is 0.7 pH units more acidic than the bulk cytosol (34), the actual $\Delta p_{\text{H}_m}$ is therefore >1 pH unit. Our data thus indicate that the resting pH$_{\text{mito}}$ is quite low in quiescent cells and increases in the wake of agonists-induced Ca$^{2+}$ signalling.

Second, we observed that changes in cytosolic pH were paralleled by changes in mitochondrial pH. Bursts of intracellular proton accumulation, due to the activity of the PMCA (see below), were readily transmitted to the mitochondrial matrix. A similar rapid equilibration of pH$_{\text{mito}}$ and pH$_{\text{cyto}}$ was reported in MDCK cells during metabolic inhibition, and attributed to the proton antiporters of mitochondria (58). Our pH$_{\text{mito}}$ recordings from permeabilized cells indicate that the matrix pH is affected both by Pi/H$^+$ symport and K$^+$/H$^+$ exchange activity (Fig. S6). Since K$^+$ and Pi are present at high concentration in the matrix and in the cytosol, these two transporters likely contribute to the rapid equilibration of the mitochondrial matrix pH during cytosolic pH excursions. Unfortunately, we could not identify pharmacologically, a single transporter that could account for the proton fluxes, and only inhibition of the mitochondrial respiratory chain consistently decreased the proton fluxes (by 20–30%) both in intact and permeabilized cells (Fig. 8). However, this inhibition probably reflects the decreased proton-motive force rather than decreased proton permeability. On the one hand, this high permeability of mitochondria to protons appears incompatible with the need for mitochondria to maintain a proton-motive force across their inner membrane. The cytosol to matrix pH gradient is essential for the import and export of a large number of metabolites. It should be kept in mind, however, that respiratory chain complexes cluster in cristae, folds of the inner mitochondrial membrane with restricted diffusional access to the rest of the intermembrane space. It is thus conceivable that H$^+$-transporters like the NHE and phosphate/H$^+$ symporter whose substrates require easy access to the cytosol might preferentially localize outside the cristae. Such an arrangement would allow generation of a large pH gradient across the cristae membrane to power the F$_{1}$F$_{0}$-ATP synthase, while accounting for the relatively high permeability of mitochondria to protons in our experiments. Our quantitative pH data also indicate that, despite the rapid adaptation of pH$_{\text{mito}}$ to changes in pH$_{\text{cyto}}$, the matrix remained at all times more alkaline than the cytosol, even during large pH excursions (Fig. 3C, ii). These data show that mitochondria can maintain a positive cytosol
to matrix pH gradient despite large variations in the absolute pH of their matrix and of their surrounding environment. This hitherto unappreciated property might be important to preserve the bioenergetic function of mitochondria in cells exposed to acid or alkaline loads.

Third, we observed unexpected biphasic changes in mitochondrial pH during stimulation of cells with a Ca\(^{2+}\)-mobilizing agonist. [Ca\(^{2+}\)\(_{\text{cyt}}\) elevations were associated with a massive acidification, whereas removal of the agonist increased pH\(_{\text{mito}}\) above pre-stimulatory levels. As a result of the overshoot, ΔpH\(_{\text{m}}\) strongly depended on prior exposure of cells to exogenous stimuli and increased by ~0.2 pH units after three successive agonist stimulations. This effect was most likely due to the Ca\(^{2+}\)-dependent activation of matrix dehydrogenases, as matrix alkalisation was also observed in permeabilized cells exposed to 10 µM Ca\(^{2+}\). The Ca\(^{2+}\)-induced alkalization, however, was of smaller amplitude than the acidification evoked by a concomitant decrease in bath pH from 7.4 to 7.2, a condition that mimics the changes occurring in intact cells stimulated with Ca\(^{2+}\)-mobilizing agonists. These data indicate that mitochondria slowly alkalinize in response to [Ca\(^{2+}\)\(_{\text{cyt}}\) elevations, but that the alkalinization is masked by a larger and faster acidification as the cytosolic pH drops concomitantly during stimulations with Ca\(^{2+}\)-mobilizing agonists.

A prominent mitochondrial acidification was reported in cultured cortical neurons stimulated with glutamate (43), and in endothelial cells stimulated with histamine (62), but not in an earlier pH\(_{\text{mito}}\) study of HeLa cells (42). We now provide a mechanistic explanation for this [Ca\(^{2+}\)\(_{\text{cyt}}\)-dependent mitochondrial acidification. Three lines of evidence indicate that the PMCA is the source of the acid produced during [Ca\(^{2+}\)\(_{\text{cyt}}\) elevations: 1) the acidification was proportional to the total calcium load, a kinetic property expected for a Ca\(^{2+}\)/H\(^+\) transporter that couples the extrusion of Ca\(^{2+}\) ions to proportional entry of protons into cells; 2) the acidification did not require Ca\(^{2+}\) uptake or Ca\(^{2+}\) release from intracellular stores, but was related to plasma membrane Ca\(^{2+}\) fluxes; 3) the acidification was prevented by La\(^{3+}\) or by alkaline pH\(_{\text{cyt}}\), two treatments that inhibit the PMCA. The PMCA mediates cytosolic acidification upon toxic stimulation of neurons with domoate (63) and has been postulated to mediate glutamate-induced acidification (43). Our data indicate that the acid generated by the PMCA is transmitted to the mitochondrial matrix. This mechanism might protect cells from excessive Ca\(^{2+}\) elevations because an acidic pH\(_{\text{mito}}\) inhibits the Ca\(^{2+}\)-induced opening of the permeability transition pore (64,65) and reduces mitochondrial production of potentially toxic radical oxygen species (31). In energized mitochondria however, an acidic pH has the opposite effect and promotes the opening of the permeability transition pore (60). Thus, whether the transmission of the acid generated by the PMCA to mitochondria has protective or deleterious effects remains to be determined.

Fourth, we report here the first estimates of the pH buffering capacity of mitochondria in intact cells. This parameter was derived from the pH changes evoked by the addition of known concentrations of membrane-permeable weak acid or bases. The determination of β\(_{\text{mito}}\) enabled us to calculate the fluxes of protons within mitochondria. Our β\(_{\text{mito}}\) values of 10-18 mM are notably lower than the ~110mM measured by phosphate-NMR in perfused liver (66), but consistent with values from isolated mitochondria of ~22 nmol/mg protein (67) that translate into a β\(_{\text{mito}}\) of 11-13 mM assuming a matrix water volume of 1.6-2.0 µl per mg of protein (68). Our values reflect the intrinsic pH buffering capacity of mitochondria since our solutions were devoid of bicarbonate. Overall, the pH dependence of the β\(_{\text{mito}}\) and β\(_{\text{cyto}}\) had a similar apparent affinity for protons, suggesting that similar molecules buffer protons within the two compartments. This is not too surprising, considering that intrinsic pH buffers (i.e. non-bicarbonate) are mainly provided by phosphate groups and side chains of amino acids (69). Importantly, the mitochondrial buffering power dropped rapidly when pH\(_{\text{mito}}\) increased from 7.6 to 8.0. This suggests that the titrable groups that can bind protons within the matrix of mitochondria have a near-neutral pKa. The sharp decrease in β\(_{\text{mito}}\) at alkaline pH facilitates the generation of a proton gradient as mitochondria become more alkaline, because fewer protons need to be pumped to generate an equivalent change in pH\(_{\text{mito}}\). The low buffering capacity of mitochondria thus facilitates the H\(^+\) -coupled entry or extrusion of mitochondrial substrates and metabolites during cell activation. On the other hand, the low β\(_{\text{mito}}\)
renders mitochondria vulnerable to surges of cytosolic acid production as a relatively small influx of protons markedly decreases pH_mito in alkaline mitochondria. This poor protection was experimentally verified to underlie a larger decrease in pH_mito than pH_cyt and a consequent decrease of ΔpH_m during Ca^{2+}-induced acidification.

In summary, we show that the mitochondrial pH is not as stable or as alkaline as generally assumed, but is dynamically regulated and can vary widely during physiological stimulations. During [Ca^{2+}]_cyt elevations, the acid generated by the plasma membrane Ca^{2+} ATPase is transmitted to the mitochondrial matrix and decreases ΔpH_m. The drop in ΔpH_m is due to the reduced buffering power of mitochondria in the alkaline pH range, which by amplifying the changes in pH_mito facilitates the generation but also the dissipation of the proton gradient.

**FOOTNOTES**

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The abbreviations used are: 2-APB – 2-aminoethoxyl diphenylborate, ANT – adenine nucleotide translocate [Ca^{2+}]_mit – mitochondrial free ionic Ca^{2+}, NCX – Na^{+}/Ca^{2+}-exchanger, pH_cyt – cytosolic pH, NHE – Na^{+}/H^{+}-exchanger, pH_cyt – cytosolic pH, pH_m – mitochondrial matrix pH. ΔpH_m – mitochondrial proton gradient, PMCA – plasma membrane Ca^{2+} ATPase, SERCA – sarco-endoplasmic reticulum Ca^{2+} ATPase.

**FIGURES LEGENDS**

**Figure 1. SypHer characterization.** A. Changes in HyPer and SypHer ratio evoked by an alkaline load (30 mM NH_{4}Cl) and by H_{2}O_{2} (200 µM) B. Confocal images of mitoSypHer (green) in fixed cells stained for TOM20 (upper red) or live cells labelled with MitoTracker Red CMXRos (lower red). Merged images (right) show clear mitochondrial targeting of mitoSypHer. Scale bars are 5 µm (upper) and 10 µm (lower). C. Excitation spectra of mitoSypHer in situ, at 37°C using a 505 nm dichroic and 535/25 emission filter. D. In situ calibration of mitoSypHer. Inset: dynamic range at physiological pH. E. Average resting pH_cyt and pH_mito measured by single-cell (left box) and high-throughput fluorescence imaging. Boxes show quartiles with circles centered on means, whiskers are SEM for the individual cells and 95% intervals for high throughput data.

**Figure 2. Mitochondria acidify during [Ca^{2+}]_cyt elevations.** A. [Ca^{2+}]_cyt responses to histamine measured with fura-2 (green, raw ratio) in cells expressing mitoSypHer (black, raw ratio). Traces show mean response of 7 cells in a single field of view with inset of simultaneous responses from a single cell. B. Parallel pH_mito (mitoSypHer, black, mean of 11 cells) and pH_cyt (cytoSypHer, blue, mean of 4 cells) acidification following 30µM histamine addition. Scale bars = 10 µM.

**Figure 3: ΔpH_m changes during and after [Ca^{2+}]_cyt elevations.** A. Simultaneous pH_mito (black, mitoSypHer) and pH_cyt (blue, SNARF) measurements in cells repeatedly stimulated with histamine (30 µM). For each cell, the mitochondrial pH gradient (ΔpH_m) was estimated as pH_mito – pH_cyt (red). Diamonds and circles are mean ± SD from 74 cells. B. Typical mitoSypHer (top, λ_ex 480 nm) and SNARF fluorescence images (bottom, λ_em 580 nm). C. Average changes in (i) pH_cyt, (ii) pH_mito, and (iii) ΔpH_m during each histamine addition and in (iv) ΔpH_m after histamine washout (mean ± SE of 74 cells). * p<0.05 for repeated measures ANOVA.
Figure 4. Ca\(^{2+}\)-dependency of the decreases in mitochondrial and cytosolic pH. A. Simultaneous [Ca\(^{2+}\)]\(_{\text{cyt}}\) (i, fura) and pH\(_{\text{mito}}\) (ii, mitoSypHer) recordings illustrate that the change in mitoSypHer ratio (iii, black) closely match the instantaneous integral of the fura-2 ratio (iii, green). Traces taken from Fig 2A. B. Effect of extracellular Ca\(^{2+}\) removal and restitution on (i) [Ca\(^{2+}\)]\(_{\text{cyt}}\) (YC3.6 cameleon, 4 cells), (ii) pH\(_{\text{mito}}\) (11 cells) and (iii) pH\(_{\text{cyto}}\) (9 cells) responses evoked by histamine (30 µM) and thapsigargin (1 µM). Insets show kinetics of SERCA-independent (i) Ca\(^{2+}\)\(_{\text{cyt}}\) clearance and (ii) pH\(_{\text{mito}}\) recovery.

Figure 5. PMCA mediates the Ca\(^{2+}\)-dependent decreases in pH\(_{\text{cyto}}\) and pH\(_{\text{mito}}\). A. La\(^{3+}\) (5 mM) blocks clearance of a [Ca\(^{2+}\)]\(_{\text{cyt}}\) elevation (i) and decrease in pH\(_{\text{mito}}\) (ii) evoked by histamine (30 µM) when SERCA is blocked with thapsigargin (Tg, 1 µM). B. Effect of La\(^{3+}\) (5 mM) on the clearance of [Ca\(^{2+}\)]\(_{\text{cyt}}\) elevations (i) and on decreases in pH\(_{\text{mito}}\) evoked by Ca\(^{2+}\) re-addition to cells treated with histamine and thapsigargin in low extracellular Na\(^{+}\) to block Ca\(^{2+}\) clearance by the Na+/Ca\(^{2+}\)-exchanger. C. Effect of alkaline pH\(_{o}\) (8.8) on [Ca\(^{2+}\)]\(_{\text{cyt}}\) (YC3.6 cameleon, 4 cells) recovery (i) after histamine removal and the decrease in pH\(_{\text{cyto}}\) (SNARF) and pH\(_{\text{mito}}\) (mitoSypHer) (ii) evoked by histamine. La\(^{3+}\) and alkaline pH\(_{o}\) both prevent Ca\(^{2+}\) clearance and prevent the decreases in cytosolic and mitochondrial pH.

Figure 6. Low mitochondrial buffering power at alkaline pH\(_{\text{mito}}\) underlies the loss of ∆pH\(_{\text{m}}\) during Ca\(^{2+}\) elevations. A. (i) Protocol used to calculate H\(^{+}\)-buffering power (β) by step-wise addition/removal of permeant weak acid. Black traces: mitoSypHer, blue traces: SNARF. Amiloride (100 µM), rotenone (5 µM), antimycin (5 µM) and oligomycin (5 µg/ml) were added to prevent membrane H\(^{+}\) transport. (ii) pH-dependence of intrinsic H\(^{+}\)-buffering power (β\(_{\text{cyto}}\) and β\(_{\text{mito}}\)). β values were binned every 0.1 pH units (mean ± SE). Dotted lines are β at mean resting pH\(_{\text{cyto}}\) and pH\(_{\text{mito}}\). Solid lines are fitted sigmoid functions. B. Calculation of proton flux (jH\(^{+}\)) for a given histamine-mediated pH change, where β is a function of pH. C. (i) Changes in pH\(_{\text{mito}}\) as a function of the changes in pH\(_{\text{cyto}}\) in the same cell during histamine responses. Regression of δpH\(_{\text{mito}}\) against δpH\(_{\text{cyto}}\) has a slope >1 reflecting the loss of ∆pH\(_{\text{m}}\) during stimulation. (ii) Mitochondrial vs. cytosolic proton fluxes. Regression of the calculated jH\(^{+}\)\(_{\text{mito}}\) vs. jH\(^{+}\)\(_{\text{cyto}}\) has a slope < 1, indicating that mitochondria resist cytosolic H\(^{+}\) fluxes. Regressions were constrained to pass through the origin.

Figure 7. Opposite effects of Ca\(^{2+}\) and H\(^{+}\) on mitochondrial matrix pH. A. pH\(_{\text{mito}}\) responses evoked by the addition of H\(^{+}\) and Ca\(^{2+}\) to permeabilized cells. Bath pH was transiently decreased from 7.4 to 7.2, then 10 µM Ca\(^{2+}\) added at pH 7.4 or during the pH switch to mimic the changes occurring in intact cells. B. Averaged pH\(_{\text{mito}}\) responses evoked in permeabilized cells by a decrease in environmental pH to 7.2, by the addition of 10 µM Ca\(^{2+}\) at pH 7.4, or by the addition of 10 µM Ca\(^{2+}\) during a switch to pH 7.2.

Figure 8. Contributions of mitochondrial transporters to mitochondrial proton fluxes. A. Effects of inhibitors of the ETC (5 µM rotenone, 5 µM antimycin ± 5 µg/ml oligomycin) and of the mitochondrial NCX (10 µM CGP-37157) on histamine-mediated pH\(_{\text{mito}}\) (black, mitoSypHer) and pH\(_{\text{cyto}}\) (blue, SNARF) responses in intact cells. Bar graphs show mean ± SE drug effects for pH\(_{\text{cyto}}\) (blue) and pH\(_{\text{mito}}\) (grey) acidification and loss of ∆pH\(_{\text{m}}\) (pink). n in bars and p-values shown for t-tests. B. Effects of inhibitors of the ETC and of the mitochondrial NCX (as above) on the pH\(_{\text{mito}}\) responses evoked by the addition of H\(^{+}\) to permeabilized cells. Bath pH was transiently decreased from 7.4 to 7.2 before and after addition of the inhibitors. Bar graphs show mean ± SE decrease in pH\(_{\text{mito}}\) evoked by the pH switch from 7.4 to 7.2. C. Average effect of the inhibitors on the jH\(^{+}\)\(_{\text{mito}}\) / jH\(^{+}\)\(_{\text{cyto}}\) flux ratio measured in intact cells during the 3\(^{rd}\) histamine response, using the 2\(^{nd}\) histamine response as internal control. Bars are mean ± SE from n cells (number in bars) from ≥ 6 coverslips. * Significantly different from control by Dunnett’s test with groupwise α = 0.05. D. Average effect of the inhibitors on the jH\(^{+}\)\(_{\text{mito}}\) flux measured in permeabilized cells during a pH switch from 7.4 to 7.2. *2 Significantly different (p < 0.05) by One-sample T-test from internal control in the absence of inhibitor.
Table 1. In vitro characterization of SypHer sensitivity to various ions and oxidizing and reducing agents. Cell lysates from SypHer expressing cells were prepared in (mM): HEPES 100, NaCl 150, β-mercaptoethanol 0.5, pH 7.00 (n=3) and 7.45 (n=3).

| Compound          | Concentration | Fold change in ratio (490/440) at pH 7.00 | Fold change in ratio (490/440) at pH 7.45 |
|-------------------|---------------|------------------------------------------|------------------------------------------|
| NaOH (pH 7.00 to 7.76) | 40mM          | 2.19±0                                    | -                                        |
| NO (SNAP)         | 0.1mM         | 0.99±0.005                                | 1.00 ± 0.002                            |
| DTT               | 1.0mM         | 0.99±0.004                                | 1.00 ± 0.004                            |
| H₂O₂             | 0.1mM         | 1.00±0.004                                | 0.99 ± 0.001                            |
| Ca²⁺              | 1.0mM         | 1.01±0.006                                | 1.00 ± 0.006                            |
| PO₄⁻              | 1.0mM         | 1.01±0.009                                | 1.00 ± 0.004                            |
References

1. Kroemer, G., Galluzzi, L., and Brenner, C. (2007) Physiol Rev 87, 99-163
2. Szabadkai, G., and Duchen, M. R. (2008) Physiology (Bethesda) 23, 84-94
3. Hoth, M., Fanger, C. M., and Lewis, R. S. (1997) J Cell Biol 137, 633-648
4. Frieden, M., Arnaudeau, S., Castelbou, C., and Demaurex, N. (2005) J Biol Chem 280, 43198-43208
5. Arnaudeau, S., Kelley, W. L., Walsh, J. V., Jr., and Demaurex, N. (2001) J Biol Chem 276, 29430-29439
6. Malli, R., Frieden, M., Osibow, K., Zoratti, C., Mayer, M., Demaurex, N., and Graier, W. F. (2003) J Biol Chem 278, 44769-44779
7. Demaurex, N., Poburko, D., and Frieden, M. (2009) Biochim Biophys Acta 1787, 1383-1394
8. Hajnoczky, G., Robb-Gaspers, L. D., Seitz, M. B., and Thomas, A. P. (1995) Cell 82, 415-424
9. Satrustegui, J., Pardo, B., and Del Arco, A. (2007) Physiol Rev 87, 29-67
10. Contreras, L., Gomez-Puertas, P., Iijima, M., Kobayashi, K., Saheki, T., and Satrustegui, J. (2007) J Biol Chem 282, 7098-7106
11. Talbot, J., Barrett, J. N., Barrett, E. F., and David, G. (2007) J Physiol 579, 783-798
12. Nicholls, D. G. (1974) Eur J Biochem 50, 305-315
13. Mitchell, P. (1961) Nature 191, 144-148
14. Nicholls, D. G. (2008) Biochim Biophys Acta 1777, 550-556
15. Wiedenmann, A., Dimroth, P., and von Ballmoos, C. (2008) Biochim Biophys Acta 1777, 1301-1310
16. von Ballmoos, C., Wiedenmann, A., and Dimroth, P. (2009) Annu Rev Biochem 78, 649-672
17. Bernardi, P. (1999) Physiol Rev 79, 1127-1155
18. Dash, R. K., and Beard, D. A. (2008) J Physiol 586, 3267-3285
19. Palty, R., Silverman, W. F., Hershfinkel, M., Caporale, T., Sensi, S. L., Parnis, J., Nolte, C., Fishman, D., Shoshan-Barmatz, V., Herrmann, S., Khananshvili, D., and Sekler, I. Proc Natl Acad Sci U S A 107, 436-441
20. Nowikovsky, K., Froschauer, E. M., Zsurka, G., Samaj, J., Reipert, S., Kolisek, M., Wiesenberger, G., and Schweyen, R. J. (2004) J Biol Chem 279, 30307-30315
21. Zotova, L., Aleschko, M., Sponder, G., Baumgartner, R., Reipert, S., Prinz, M., Schweyen, R. J., and Nowikovsky, K. J Biol Chem 285, 14399-14414
22. Jiang, D., Zhao, L., and Clapham, D. E. (2009) Science 326, 144-147
23. Scarpa, A., and Azone, G. F. (1970) Eur J Biochem 12, 328-335
24. Rottenberg, H., and Scarpa, A. (1974) Biochemistry 13, 4811-4817
25. Wingrove, D. E., Amatruda, J. M., and Gunter, T. E. (1984) J Biol Chem 259, 9390-9394
26. Bernardi, P., and Azone, G. F. (1983) Eur J Biochem 134, 377-383
27. Nicholls, D. G., and Chalmers, S. (2004) J Bioenerg Biomembr 36, 277-281
28. Mitchell, P., and Moyle, J. (1969) Eur J Biochem 7, 471-484
29. Chen, L. B. (1988) Annu Rev Cell Biol 4, 155-181
30. Lambert, A. J., and Brand, M. D. (2004) Biochem J 382, 511-517
31. Selivanov, V. A., Zeak, J. A., Roca, J., Cascante, M., Trucco, M., and Votyakova, T. V. (2008) J Biol Chem 283, 29292-29300
32. Hoek, J. B., Nicholls, D. G., and Williamson, J. R. (1980) J Biol Chem 255, 1458-1464
33. Brand, M. D., and Felber, S. M. (1984) *Biochem J* **217**, 453-459
34. Porcelli, A. M., Ghelli, A., Zanna, C., Pinton, P., Rizzuto, R., and Rugolo, M. (2005) *Biochem Biophys Res Commun* **326**, 799-804
35. Halestrap, A. P., and Pasdois, P. (2009) *Biochim Biophys Acta* **1787**, 1402-1415
36. Brand, M. D., and Esteves, T. C. (2005) *Cell Metab* **2**, 85-93
37. Jousset, H., Frieden, M., and Demaurex, N. (2007) *J Biol Chem* **282**, 11456-64
38. Belousov, V. V., Fradkov, A. F., Lukyanov, K. A., Staroverov, D. B., Shakhbazov, K. S., Terskikh, A. V., and Lukyanov, S. (2006) *Nat Methods* **3**, 281-286
39. Palmer, A. E., Jin, C., Reed, J. C., and Tsien, R. Y. (2004) *Proc Natl Acad Sci U S A* **101**, 17404-17409
40. De Marchi, U., Campello, S., Szabo, I., Tombola, F., Martinou, J. C., and Zoratti, M. (2004) *J Biol Chem* **279**, 37415-37422
41. Roos, A., and Boron, W. F. (1981) *Physiol Rev* **61**, 296-434
42. Abad, M. F., Di Benedetto, G., Magalhaes, P. J., Filippin, L., and Pozzan, T. (2004) *J Biol Chem* **279**, 11521-11529
43. Bolshakov, A. P., Mikhailova, M. M., Szabadkai, G., Pinelis, V. G., Brustovetsky, N., Rizzuto, R., and Khodorov, B. I. (2008) *Cell Calcium* **43**, 602-614
44. Matsuyama, S., Llopis, J., Deveraux, Q. L., Tien, R. Y., and Reed, J. C. (2000) *Nat Cell Biol* **2**, 318-325
45. Scaduto, R. C., Jr., and Grottyhann, L. W. (1999) *Biophys J* **76**, 469-477
46. Wiederkehr, A., Park, K. S., Dupont, O., Demaurex, N., Pozzan, T., Cline, G. W., and Wollheim, C. B. (2009) *EMBO J* **28**, 417-428
47. Trenker, M., Malli, R., Fertschai, I., Levak-Frank, S., and Graier, W. F. (2007) *Nat Cell Biol* **9**, 445-452
48. Frieden, M., James, D., Castelbou, C., Danckaert, A., Martinou, J. C., and Demaurex, N. (2004) *J Biol Chem* **279**, 22704-22714
49. Niggli, V., Sigel, E., and Carafoli, E. (1982) *J Biol Chem* **257**, 2350-2356
50. Thomas, R. C. (2009) *J Physiol* **587**, 315-327
51. Niggli, V., and Sigel, E. (2008) *Trends Biochem Sci* **33**, 156-160
52. Brini, M., and Carafoli, E. (2009) *Physiol Rev* **89**, 1341-1378
53. Carafoli, E. (1992) *J Biol Chem* **267**, 2115-2118
54. Gover, T. D., Moreira, T. H., Kao, J. P., and Weinreich, D. (2007) *Cell Calcium* **41**, 389-396
55. Vaughan-Jones, R. D., and Wu, M. L. (1990) *J Physiol* **425**, 429-448
56. Santo-Domingo, J., and Demaurex, N. *Biochim Biophys Acta*
57. Llopis, J., McCaffery, J. M., Miyawaki, A., Farquhar, M. G., and Tsien, R. Y. (1998) *Proc Natl Acad Sci U S A* **95**, 6803-6808
58. Balut, C., vandeVen, M., Despa, S., Lambrichts, I., Ameloot, M., Steels, P., and Smets, I. (2008) *Kidney Int* **73**, 226-232
59. Bernardi, P., Vassanelli, S., Veronese, P., Colonna, R., Szabo, I., and Zoratti, M. (1992) *J Biol Chem* **267**, 2934-2939
60. Kristian, T., Bernardi, P., and Siesjo, B. K. (2001) *J Neurotrauma* **18**, 1059-1074
61. Halestrap, A. P. (2009) *J Mol Cell Cardiol* **46**, 821-831
62. Malli, R., Frieden, M., Osibow, K., and Graier, W. F. (2003) *J Biol Chem* **278**, 10807-10815.
63. Vale-Gonzalez, C., Alfonso, A., Sunol, C., Vieytes, M. R., and Botana, L. M. (2006) *J Neurosci Res* **84**, 326-337
64. Petronilli, V., Cola, C., and Bernardi, P. (1993) *J Biol Chem* **268**, 1011-1016
65. Bernardi, P. (1992) *J Biol Chem* **267**, 8834-8839
66. Durand, T., Delmas-Beauvieux, M. C., Canioni, P., and Gallis, J. L. (1999) *Cryobiology* **38**, 68-80
67. Mitchell, P., and Moyle, J. (1965) *Nature* **208**, 147-151
68. Bednarczyk, P., Barker, G. D., and Halestrap, A. P. (2008) *Biochim Biophys Acta* **1777**, 540-548
69. Casey, J. R., Grinstein, S., and Orlowski, J. *Nat Rev Mol Cell Biol* **11**, 50-61
Figure 1, Poburko et al.
Figure 4, Poburko et al.
Figure 5, Poburko et al.
Figure 6, Poburko et al.

A. i. ETC / NHE inhibition

butyric acid (mM)

7.0 7.2 7.4 7.6 7.8
pH

ETC / NHE inhibition

20 10 5 2.5 1
5 min

B. δpH_mito

δpH_cyto

hist

m = 1.45 ± 0.04 > 1
r² = 0.25

jH⁺_cyto (mM)

jH⁺_mito (mM)

C. ii. jH⁺_cyto (mM)

m = 0.89 ± 0.03 < 1
r² = 0.70

\[ jH^+ = \delta pH \times \beta_{mid} \]
