Azide- and Vanadate-Sensitive M-Phase Alkalinity and Cytosolic Acidification of Chang Liver Cells

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ABSTRACT—Flow cytometric cell-by-cell evaluation of NH₄Cl acidification of human Chang cells showed that at steady state, 3% of the cells remained alkalinized (pH > 7.0) over an extended period (up to 80 min) despite the absence of extracellular Na⁺ and HCO₃⁻. In fluorescence microscopy, the acidification-resistant cells were characteristically rounded M-phase cells. Both mean cytosolic pH and M-phase alkalinity were however sensitive to (a) azide and oligomycin, inhibitors of F-ATPase (ATP synthase), and to (b) vanadium ions, the phosphate analogue of P-ATPase (ATP-hydrolyzing), in dose-dependent and time-dependent manners. Dead cell indices were constant at ~10%. Thiocyanate chaotrophic anions, which cleave the V-ATPase structure, had no effect. Since ATP synthesizing F-ATPase (ATP synthase) is coupled to ATP-hydrolyzing P-ATPase as 'master-&-slave', azide- and oligomycin-sensitivity corroborated with vanadate-sensitivity in suggesting energized proton pumping modulating (a) M-phase alkalinity and (b) cytosolic pH, against acidification.

Keywords: Flow cytometric evaluation (BCECF ratio), Proton pumping (M-phase and interphase), ATPase sensitivity (F-ATPase and P-ATPase)

Intracellular pH (pHᵢ) modulation seems implicated in virtually every biological process, including hyperproliferative cancer states. The Na⁺-dependent Na⁺/H⁺ antiporter is perceived as the main regulator of cytosolic pH (pHᵢ) and the mechanistic causation of M-phase alkalinity. More recently, involvement of H⁺-translocating ATPases (proton pumps) has been suggested but only in specialized cell types, viz. acid-secreting osteoclasts and phagocytes, and implicating the participation of V-ATPases that are insensitive to vanadate (1, 2).

Here we show, in cell-by-cell analysis using flow cytometry, that cytosolic acidification of human Chang liver cells subjected to continuous incubation in acidic (pH 5.5) NH₄Cl without extracellular Na⁺ and HCO₃⁻ was enhanced by (a) azide and oligomycin, F-ATPase inhibitors (3), and (b) the P-ATPase inhibitor vanadate (4), in dose-dependent and time-dependent manners. We further show, in conjunction with fluorescence microscopy, that the NH₄Cl-induced acidification cannot quench M-phase alkalinity, tested up to 80 min of incubation time, but was nevertheless also sensitive to azide, oligomycin and vanadate in similar dose- and time-dependent manners. Chaotrophic SCN⁻ anions from potassium thiocyanate (5) could cleave the V-ATPase and produce loss of ATPase activity and associated proton pumping (6). However, we found KSCN to have no effect on our system when tested at concentrations of 100 nM, 1 µM, 10 µM, 100 µM, 1 mM and 10 mM. The dead cell index of cells suspended in NH₄Cl with or without inhibitors was constant at ~10%.

Monolayer cultures of Chang liver cells (ATCC CCL 13) were grown in Dulbecco’s modified Eagle’s medium (DMEM; Sigma, St. Louis, MO, USA) with 10% fetal calf serum (Cytosystems, Castle Hill, Australia). Parallel cultures were seeded in 25 cm² flasks (Costar, Cambridge, MA, USA) at 3 × 10⁵ cells/flask from a stirred stock suspension and grown to near confluency.

For measurement of pHᵢ, the cells were loaded with 10 µM BCECF-am (Molecular Probes, Eugene, OR, USA) as previously described (7). BCECF-loaded monolayer cells were scrapped with a Cell Scraper (Costar) and triturated by syringing in a 5-ml syringe with a 22-gauge needle to obtain a single cell suspension for flow cytometry. Cells were rinsed in NH₄Cl buffer (50 mM NH₄Cl, 50 mM glucose, which had a pH of 5.5, unadjusted; the RTF (round-to-flat) solution used previously by us for cell-acidifications to reflatten rounded alkalinized cells (7)). The cell suspension in NH₄Cl buffer was kept stirred in a thermostated (37°C) cuvette for time-responses. Aliquots...
of the incubated cell suspension were evaluated at intervals in the EPICS Profile II flow cytometer. Experimental cell suspensions contained (a) sodium azide (NaN₃, mol wt. 65.01; Merck, Darmstadt, FRG), (b) vanadate (IV) oxide sulphate pentahydrate, (O₂SV·5H₂O, mol wt. 253.08; Fluka Chemica, Buchs, Switzerland), (c) oligomycin (A, B and C, Sigma 0-4876, stock solution is 20 μg/ml of DMSO) and (d) potassium thiocyanate (KSCN, mol wt. 97.18; Sigma) in the NH₄Cl buffer. Control samples were without the ATPase inhibitors. Dose-responses were done with cell suspensions containing various concentrations (a) azide (0 to 10 mM), (b) oligomycin (0 to 1 mg/ml), (c) vanadate (0 to 2 mM) and (d) potassium thiocyanate (0 to 10 mM). Concurrent dead cell indices using trypan blue dye uptake in the respective solutions were evaluated as previously described (8).

For calibration of pH to flow cytometer FL1/FL3 channel ratios (see inset in Fig. 1A(i-ii)), BCECF-loaded cells were suspended in KCl-nigericin buffer (40 mM HEPES, 115 mM KCl, 1 mM MgCl₂ and 10 μM nigericin; Sigma) (9) which allowed equilibration between pHᵢ and pHₑo.

For fluorescence microscopy, BCECF-loaded cells were processed in situ. Incubations in NH₄Clbuffer with and without azide or vanadate were as monolayers. After incubation and air-drying, the cells were examined with a Univar epifluorescence microscope (Reichert-Jung, Vienna, Austria) as previously described (10).

Settings of the EPICS Profile II flow cytometer (Coulter Electronics, Hialeah, FL, USA), equipped with the PowerPak option, were: (a) 15 mW 488 nm argon-gas laser light source, (b) dual wavelength fluorescence emission at 525 nm and 635 nm (11), and (c) 2-min sampling time, at a flow rate of 15 μl/min. Ratioed histograms and analysis were obtained in real time during the run. Histogram overlays were generated by the EPICS Elite Flow Cytometer Workstation program, version 3.0 (Coulter).

Incubating human Chang liver cells in acidic (pH 5.5) NH₄Cl buffer caused the intracellular pH (pHᵢ) to drop from 7.1 (the resting level, solid line trace in Fig. 1C(i)) to pH 6.6 within 2 min (dotted line trace in Fig. 1A(i)). Even so, over 20% of the cells remained unacidified with > pH 7 (tail area of histogram beyond the vertical bar, pH 7 intercept). Adding vanadium (IV) ions or azide into the NH₄Cl incubation buffer enhanced the decline in mean cytosolic pH in a dose-dependent manner (Fig. 1A(i-iv), 1B(i-iv)). Concurrently, there was also a dose-dependent decline in the percentage of cells with > pH 7 (Fig. 1A(i-iv), 1B(i-iv), v). The percentage of > pH 7 cells dropped from over 20% to well below 10% when the added vanadate concentration reached 200 μM or added azide concentrations reached 10 mM azide. Dead cell indices obtained from trypan blue dye uptake by samples of these cells were about 10% in NH₄Cl buffer alone, and remained at that level with added ATPase inhibitors (see dashed line in Fig. 1A(v) and Fig. 1B(v)).

Time-responses with continuous incubation of the cells in NH₄Cl buffer alone showed that the decline in mean cytosolic pH reached a steady state after 40 min, maintaining at pH 6.2 up to 80 min (Fig. 1C(i-iii), Fig. 1F(iv) control, Fig. 2A cf. 2B). At the steady state, after 40-min incubation, between 2.9% and 3.3% of cells (Fig. 1C(iii), iii) and Fig. 1F(ii) control trace), remained unacidified, with > pH 7. Fluorescence microscopy of the in situ monolayer cell state showed that cells that had higher fluorescence (higher pHᵢ) at this stage were rounded in morphology, either singly or in doublets (Fig. 2B), phenomena characterizing M-phase cells (10, 12), which we have previously shown to have lost more than 80% of their surface area relative to the flat interphase counterparts (10). Although M-phase cells are known to be more alkaline than their interphase counterparts (10), their long persistence (up to 80-min incubation time) in an acidic (pH 5.5, the pH of NH₄Cl buffer) extracellular environment that was Na⁺ free and HCO₃⁻ free, appeared remarkable. The NH₄Cl buffer used in these experiments was the RTF solution that we had previously shown to be effective, cyclically, in re-flattening interphase cells rounded by inducing intracellular alkalinization (7). It seems that M-phase cells may have distinctive proton extrusion capabilities.

Time-response with continuous incubation of the cells in NH₄Cl buffer with the addition of 200 μM vanadium (IV) oxide sulphate showed that at the steady state level, there was (a) marked reduction (67%) in the proportion of cells with > pH 7, dropping from the 3% level of the controls to between 1.3% and 0.8%, as well as (b) an enhanced depression of mean pHₑo, dropping now below the extracellular pH (pHₑo) level of 5.5 (Fig. 1D(i-iii), Fig. 1F(i-ii)). Fluorescence microscopy confirmed the decline in fluorescence of the cells, including M-phase doublets (Fig. 2C cf. Fig. 2A, 2B). All transport ATPases that are known to proceed through a phosphoenzyme intermediate (the P-type or phosphorylated ATPases) are inhibited by vanadyl ions (3-6). Cytosolic pH regulation and maintenance of M-phase alkalinity that responded to vanadate in dose-dependent and time-dependent manners in non-specialized mammalian cells appear novel.

The time-response with continuous incubation of cells in NH₄Cl buffer with the addition of 10 mM sodium azide showed that at the steady state level after 40 min, there was (a) a virtual abolition (92% reduction) of > pH 7 cells which became reduced to baseline proportions, between 0.3% and 0.2%, and (b) enhanced decline in mean pHₑo, falling well below pHₑo to 4.6-4.7 (Fig. 1E(i-ii), Fig. 1E(i-ii)). Figure 2D shows that M-phase doublets had acidified (low BCECF fluorescence) after 40-min incubation with azide. Azide is an inhibitor of mitochondrial
Fig. 1. Flow cytometric cell-by-cell evaluation of intracellular pH (BCECF ratios) in human Chang liver cells incubated in 50 mM NH₄Cl-glucose: (A) and (B) are dose-responses at 2-min pulses of the inhibitors, vanadyl (IV) sulphate and sodium azide. Controls are without the inhibitors. Time response without inhibitors, (C); with vanadyl (IV) sulphate, (D); and with sodium azide, (E) are line-plotted in (F). The oligomycin dose-response is shown in (G).
Fig. 2. Fluorescence microscopy of BCECF-loaded human Chang liver cells in situ. Higher fluorescence intensity indicates higher intracellular pH (see calibration curve, inset in Fig. 1A(iv)).

(A) Before NH₄Cl incubation. (B) After 40-min NH₄Cl incubation without azide or vanadate. (C) After 40-min NH₄Cl incubation with 200 μM vanadyl (IV) oxide sulphate. (D) After 40-min NH₄Cl incubation with 10 mM sodium azide. Arrows show rounded doublets of M-phase cells. Whether as single cells or doublets, the rounded M-phase cells are brightly fluorescent in (A) and (B) but less so in (C) where the fluorescence of a number of doublets appear reduced in the presence of vanadate. In (D) M-phase cells are hardly discernible because of poor fluorescence in the presence of azide. ×10 obj.

(F-type) ATPase (1, 3, 4). In higher eukaryotic cells, all ATPase are coupled by ATP in a 'master-&-slave' relationship where the mitochondrial (F-type) ATPase functions obligatorily in the direction of ATP synthesis (the master) supplying ATP to V- and P-ATPases (the slaves) that function obligatorily in the direction of ATP hydrolysis to perform the cellular work (5). Accordingly then, inhibition of the master (F-ATPase) meant inhibition of ATP synthesis. The consequential ATP depletion would downregulate the slave ATPases. Sensitivity of cytosolic pH and M-phase alkalinity to F-ATPase inhibition was further corroborated by significant dose-dependent responses to oligomycin (Fig. 1G(i–iii)), another F-ATPase inhibitor for which the name Fₒ-ATPase is given.

Recently, the externalization of vacuolar (V-type) proton pumps onto the plasma membrane surface via Ca²⁺-dependent exocytosis, following CO₂-induced acidification in the acid-secreting turtle bladder epithelium, has been cited as a new mechanism for the regulation of cellular pH (13). In that consideration, the response to acidification in human Chang liver cells without being aided by extracellular Na⁺ or HCO₃⁻, appeared different since (a) M-phase alkalinity was associated with rounding shape change with much reduced surface area, a phenomenon that had been attributed to endocytic internalization rather than exocytic externalization of the plasma membrane that would expand the cell’s surface area (7, 10); (b) azide- and oligomycin-sensitivity implicated F-ATPase participation; and (c) vanadate-sensitivity implicated P-ATPase participation that, together with F-ATPase participation, could support the suggestion of a 'master-&-slave' relationship. While the observed KSCN insensitivity, by itself, may not necessarily exclude the participation of V-ATPase, nevertheless, vanadate-sensitive dose- and time-dependent responses (see Figs. 1A, 1D and 1F) would still be inconsistent with V-ATPase participation (3–6). This seems to refute the earlier suggestion that osteoclast V-ATPase was an example of vanadate-sensitive V-ATPase involved in proton extrusion across the plasma membrane (14) (see 1). Proton pumping by P-ATPase expression in the plasma membrane of mammalian cells has been shown to be capable of producing cytosolic alkalinization, including rounding shape change (15).

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