Stable intracellular acidification upon polyamine depletion induced by α -difluoromethylornithine or N^1,N^{12} -bis(ethyl)spermine in L1210 leukaemia cells

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Polyamines play major roles in ionic and osmotic regulation, but their exact involvement in specific ion transport processes is poorly defined. Treatment of L1210 mouse leukaemia cells with either 5 mM α -difluoromethylornithine (DFMO), a suicide substrate of ornithine decarboxylase, or 25 μ M N^1,N^{12} -bis(ethyl)spermine (BE-3-4-3), a dysfunctional polyamine analogue, caused a stable decrease in intracellular pH (pH_i) by 0.1–0.4 unit from steady-state control values between 7.4 and 7.6, as measured either by partition of a weak acid or with a fluorescent pH-sensitive probe. This effect was not related to cell growth status or differences in metabolic acid generation, and was observed in either the presence or absence of HCO_3^- . Exogenous spermidine (10–25 μ M) or putrescine (25–50 μ M) fully reversed DFMO- or BE-3-4-3-induced acidification within 2 and 8 h respectively. Recovery of pH_i in L1210 cells after a

nigericin- or NH_4^+ -mediated acid load in HCO_3^- -free buffers was mediated by Na^+/H^+ antiporter activity, in addition to a minor Na^+ -independent and amiloride-insensitive pathway. Decreased steady-state pH_i was maintained in polyamine-depleted L1210 cells after recovery from acid stress. Moreover, the pH_i -dependence of the rate of Na^+ -dependent H^+ extrusion after an acid stress was altered by DFMO and BE-3-4-3, resulting in a set-point which was lower by 0.25–0.30 pH unit in polyamine-depleted cells. On the other hand, neither the rate nor the magnitude of Na^+/H^+ -exchanger-mediated alkalization induced by hypertonic shock was decreased by polyamine depletion. Thus polyamine depletion induces a persistent defect in pH_i homeostasis which is due, at least in part, to a stable decrease in the pH_i set-point of the Na^+/H^+ exchanger.

INTRODUCTION

There is compelling evidence that putrescine and polyamines play major functions in the adaptation to ionic and osmotic stress in most living cells [1,2]. Hypo-osmotic stress is perhaps the most potent known agent for the induction of ornithine decarboxylase (ODC; EC 4.1.1.17), the highly regulated enzyme responsible for putrescine synthesis, and putrescine is an important viability factor in cells exposed to low osmolality [3–5]. Moreover, ODC is rapidly and dramatically induced by increased Na^+ influxes, concomitant with system A-catalysed amino acid uptake or Na^+ /hexose co-transport activities [6–8]. Putrescine biosynthesis is also increased upon exposure to several types of ionic insult, including acid stress, such as K^+ and Mg^{2+} deficiency, high NH_4^+ concentrations, SO_2 or low extracellular pH (reviewed in [2]). A role for increased Na^+/H^+ antiporter activity in the induction of ODC by various stimuli has also been postulated [9,10], but is still controversial [11]. Polyamines can also regulate the conductance of ion channels, such as in the case of the N -methyl-D-aspartate receptor [12] and inward rectifier K^+ channels [13].

Current understanding of the physiological functions of putrescine and polyamines in most, if not all, of these phenomena is very limited. Moreover, few studies have addressed the effect

of polyamine depletion on the regulation of specific membrane ion fluxes [14,15]. Because of the putative involvement of increased putrescine biosynthesis in environmental stresses leading to intracellular pH (pH_i) imbalance [2], we have investigated the possible consequences of polyamine depletion on steady-state pH_i , using the suicide ODC substrate α -difluoromethylornithine (DFMO), which rapidly depletes intracellular putrescine and spermidine [16,17], and N^1,N^{12} -bis(ethyl)spermine (BE-3-4-3), a cytotoxic polyamine analogue which depresses polyamine biosynthetic activity while depleting polyamines by displacement from cellular binding sites and/or metabolic conversion [18–21]. We report that polyamine depletion by both agents induces a stable decrease in steady-state pH_i , which is rapidly reversed by exogenous polyamines. This acidification is associated with a defect in the mechanism of proton extrusion after an acid load, and may result from a sustained decrease in the set-point of the Na^+/H^+ exchanger.

MATERIALS AND METHODS

Materials

DFMO and BE-3-4-3 were generously provided by the Marion-Merrell Dow Research Institute (Cincinnati, OH, U.S.A.) and Dr. Raymond J. Bergeron (University of Florida, Gainesville,

Abbreviations used: BCECF, 2',7'-bis-(2-carboxyethyl)-5-(6-carboxyfluorescein); BE-3-4-3, N^1,N^{12} -bis(ethyl)spermine; β_i , intracellular buffering capacity; DFMO, α -difluoromethylornithine; [^{14}C]DMOD, 5,5-[2- ^{14}C]dimethylloxazolidine-2,4-dione; NMG, N -methyl-D-glucamine; ODC, ornithine decarboxylase (EC 4.1.1.17); pH_i , intracellular pH.

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FL, U.S.A.). 5,5-[2-¹⁴C]Dimethyloxazolidine-2,4-dione ([¹⁴C]DMOD) (47.2 Ci/mol) and [U-¹⁴C]sucrose (4.8 Ci/mol) were purchased from Dupont-New England Nuclear (Wilmington, DE, U.S.A.). ³H₂O (0.91 μ Ci/ml) was obtained from Amersham Corp. (Arlington Heights, IL, U.S.A.). Silicone oils 550 and 200 were from the Dow Corning Corporation (Midland, MI, U.S.A.). 2',7'-Bis-(2-carboxyethyl)-5-(6)-carboxy-fluorescein (BCECF) acetoxymethyl ester was obtained from Molecular Probes Inc. (Eugene, OR, U.S.A.). BCECF acetoxymethyl ester stock solutions were prepared fresh before use at 500 μ M in tissue-culture grade DMSO (Sigma). Bafilomycin A₁ was purchased from the Kamiya Biomedical Company (Thousand Oaks, CA, U.S.A.). Amiloride, nigericin, *N*-methyl-D-glucamine (NMG) and other biochemical reagents were from Sigma.

Cell culture

L1210 mouse leukaemia cells were grown as suspension cultures in RPMI 1640 medium supplemented with 10 % (v/v) Nu-Serum (Collaborative Research Inc., Lexington, MA, U.S.A.), 16 mM Hepes and 7.8 mM Mops in a 95 % air/5 % CO₂ atmosphere with 5 % relative humidity, and were routinely passaged by a 1:10 dilution of the cell culture every 3 or 4 days. L1210 cell variants (D-R) expressing ≥ 100 -fold higher levels of ODC [22] were grown in the same medium supplemented with 10 mM DFMO. Unless otherwise indicated, drug additions were made at the time of seeding. Media were supplemented with aminoguanidine (1 mM) to inhibit serum amine oxidase activity [23] in experiments requiring addition of polyamines to the growth medium. Cell number was determined with a Coulter Counter (model ZF; Coulter Electronics, Hialeah, FL, U.S.A.).

Determination of pH_i by the weak acid distribution method

The procedure used for pH_i determination with [¹⁴C]DMOD was adapted from Strzelecki et al. [24] and Simchowicz [25]. Briefly, cells were collected by centrifugation and resuspended in buffer A [140 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 0.5 mM MgCl₂, 10 mM Hepes, pH 7.40, 0.02 % (w/v) BSA, 5.6 mM D-glucose] at a final density of (4.8–8.0) $\times 10^6$ cells/ml. Aliquots (500 μ l) of this suspension were distributed to 15 ml polypropylene round-bottom tubes and gently shaken at 37 °C for 15 min for pre-equilibration. Then 10 μ l of a [¹⁴C]DMOD solution (51 μ Ci/ml in DMSO) was added to triplicate aliquots of cell suspension for each treatment used. To another set of triplicate samples was added 10 μ l of [¹⁴C]sucrose (80 μ Ci/ml in water) for the determination of the extracellular water space. Cells were allowed to equilibrate with the labelled compounds for an additional 15 min period. Intracellular water volume was estimated by adding 10 μ l of ³H₂O (431 μ Ci/ml) to every sample 1 min before the end of the incubation. Aliquots of the cell suspension (250 μ l) were then transferred to conical 500 μ l microcentrifuge tubes containing 50 μ l of 14 % (w/v) HClO₄ overlaid with a 200 μ l cushion made of a silicone oil mixture (550 Dow-Corning silicone oil/220 Dow-Corning silicone oil, 183:17, v/v). Cells were then centrifuged for 30 s through the oil cushion, and the tube contents immediately frozen in liquid N₂. Both the bottom (containing the cell pellet and HClO₄-soluble cellular material) and the top (extracellular labelled medium) layers of each tube were excised with a scalpel, and directly transferred to a scintillation vial containing 10 ml of Formula-989 cocktail (Dupont-New England Nuclear) for radioactivity determination using a double isotope program for simultaneous ³H and ¹⁴C emission.

pH_i was calculated according to the formula:

$$\text{pH}_i = \text{pK}_a' + \log \left\{ \frac{C_i}{C_o} [1 + 10^{\text{pH}_o - \text{pK}_a'}] \right\} \quad (1)$$

where C_i and C_o represent the internal and external [¹⁴C]DMOD molar concentrations respectively and pH_o is the extracellular pH, using 6.13 as the pK_a' value for DMOD at 37 °C [25,26]. C_i was estimated as:

$$C_i = \frac{{}^3\text{H}_2\text{O}_{\text{sp}}(\text{DMOD}_{\text{pellet}} - C_o\text{EWS})}{\text{DMOD}_{\text{sp}}[{}^3\text{H}_2\text{O}_{\text{pellet}} - ({}^3\text{H}_2\text{O}_{\text{sp}})\text{EWS}]} \quad (2)$$

where ³H₂O_{sp} and DMOD_{sp} are the specific radioactivities of ³H₂O and [¹⁴C]DMOD respectively (in d.p.m./mol), ³H₂O_{pellet} and DMOD_{pellet} are the total [¹⁴C]DMOD- and ³H₂O-associated radioactivities (in d.p.m.) respectively in the pellet, and EWS is the extracellular water space, measured as the (total [¹⁴C]sucrose d.p.m. in the pellet/molar concentration of [¹⁴C]sucrose in the supernatant) ratio.

Fluorimetric determination of steady-state pH_i with BCECF

Following prior treatment with DFMO or BE-3-4-3, between 2 $\times 10^6$ and 5 $\times 10^6$ L1210 cells were pelleted by low-speed centrifugation and resuspended in 5 ml of serum-free RPMI 1640 containing 0.1 % (w/v) BSA and 2 μ M BCECF acetoxymethyl ester [27] [with 0.4 % (v/v) DMSO] and buffered with 23.8 mM NaHCO₃, 16 mM Hepes and 7.8 mM Mops to pH 7.40 $\pm$ 0.02 (at 37 °C and 5 % CO₂). Conditioned medium from the supernatant was saved for the fluorescence determination step. Cells were transferred to a 60-mm dish and loaded with the dye for 30 min in a 5 % CO₂ incubator at 37 °C. After brief centrifugation, the bright yellow cell pellet was re-dispersed in 5 ml of the original conditioned medium of the respective cell culture and incubated for 10 min to allow for gas equilibration. Cells were then quickly transferred to a 3 ml plastic cuvette (Sarstedt) which was immediately closed with a piece of Parafilm. Spectrometric determination of cellular fluorescence was then performed with either a Shimadzu RF5000U or a Spex Fluoromax programmed for alternate excitation at 455 and 505 nm (5 nm bandwidth) at 2 s intervals at a constant emission wavelength of 532.5 nm (15 nm bandwidth). Cells were briefly resuspended by gentle inversion of the cuvette prior to measurement. The ratios of fluorescence intensities measured at the two excitation wavelengths (I_{505}/I_{455}) were averaged for each sample from three successive determinations made at 10 s intervals.

Calibration of pH_i values was performed by the nigericin clamping method [28]. Briefly, 8–10 ml of an L1210 cell suspension (1 $\times 10^7$ cells/ml) loaded with BCECF acetoxymethyl ester as described above was resuspended in 400 μ l of equilibration buffer (20 mM Mes, 20 mM Mops, 135 mM KCl, 1 mM MgCl₂, pH 7.00), and 1.6 ml of the same buffer adjusted at one of eight different standard pH values (between 6.4 and 7.8) was mixed with the cell suspension. The resulting pH value was corrected for the small shift resulting from this mixing procedure. Nigericin (2.7 μ M) was then added to the suspensions and cells were incubated in glass scintillation vials for 10 min under gentle agitation at 37 °C. The I_{505}/I_{455} excitation ratio of the nigericin-treated cells was then measured; this was linear within the range of pH values used.

Determination of the rate of extracellular acidification

After a 48 h incubation in the presence or absence of drugs, cells were harvested by centrifugation and resuspended at 4.5 $\times 10^6$

cells/ml in a lightly buffered solution (140 mM NaCl, 5 mM KCl, 10 mM D-glucose, 0.1 mM Hepes, pH 7.3). Medium acidification was monitored with a standard glass electrode, and the output of the pH meter (Accumet 915; Fisher Scientific) was connected to a strip chart recorder. The sensitivity of detection was 0.010 ± 0.005 pH unit in the pH range 6.3–7.6.

Determination of pH_i after acid loading or hyperosmotic shock

Prior to these experiments, L1210 cells pretreated with DFMO or BE-3-4-3 were loaded with BCECF acetoxymethyl ester as indicated above, except that all RPMI 1640 solutions were buffered with 32 mM Hepes/15.6 mM Mops (without NaHCO_3), and cells were stored at 37 °C in tightly closed 25 cm² flasks under CO_2 -free conditions in fresh serum- and HCO_3^- -free RPMI 1640 medium [+0.1 % (w/w) BSA and DFMO or BE-3-4-3 as required].

Two methods were used to measure recovery of pH_i from an acid stress. In the first method, 10 ml of BCECF-loaded L1210 cells was centrifuged and resuspended in 2 ml of NMG (Na^+ -free) buffer [20 mM Tris/HCl, pH 7.40, 150 mM NMG, 0.42 mM $\text{Ca}(\text{NO}_3)_2$, 0.41 mM MgSO_4 , 10 mM D-glucose, 2.8 mM K_2HPO_4] in a spectrophotometric cuvette. The pH_i of the cell suspension was allowed to equilibrate for 90 s in the fluorimeter chamber under mild magnetic stirring, and 4 μl of stock nigericin solution was then added directly to the cells to a 1.4 μM final concentration. BSA [0.5 % (w/v) in NMG buffer] was added to adsorb the ionophore [29] when the desired pH_i had been reached. In order to activate the Na^+/H^+ antiporter and pH_i recovery, 2 ml of NaCl buffer (of composition identical to NMG buffer, but containing 150 mM NaCl instead of NMG) was then added to the cell suspension. pH_i was monitored until stabilization of the fluorescent signal was reached.

As a second method for acid loading, dye-loaded cells were allowed to equilibrate for 2 min in 2 ml of NaCl buffer, and 2 ml of $\text{Na}^+/\text{NH}_4^+$ buffer [of identical composition to Na^+ buffer but containing 40 mM NH_4Cl and 110 mM NaCl] was then added to induce intracellular alkalization [27]. At 2 min after the maximal pH_i was reached, NH_4^+ -loaded cells were pelleted for 10 s in a microcentrifuge and resuspended in 2 ml of NMG buffer, followed 90 s later by the addition of 2 ml of NaCl buffer to activate Na^+/H^+ exchanger activity, with continuous monitoring of the I_{505}/I_{455} excitation ratio.

For hyperosmotic stress experiments, dye-loaded cells were resuspended in 1.5 ml of NaCl or NMG buffer as indicated, and after pH_i equilibration were subjected to hypertonic shock by the addition of either sucrose (250 mM in either NaCl or NMG buffer) or an osmotically equivalent amount of NaCl (137 mM in NMG buffer). After maximal alkalization, pH_i recovery was assessed by the addition of Tris buffer (identical to NaCl buffer but without NaCl) to restore osmolality to its original value (325 mosmol/kg).

Determination of the rate of proton efflux

The H^+ extrusion capacity as a function of pH_i was determined after inducing various levels of intracellular acidification with nigericin as described above. The rate of H^+ efflux (in $\text{mol} \cdot \text{min}^{-1} \cdot \text{l}^{-1}$) was determined from the initial rate of pH_i recovery ($\Delta p\text{H}_i/\Delta t$) multiplied by the intracellular buffering capacity (β_i). β_i was determined for each experimental treatment by resuspending BCECF-loaded cells in NMG buffer, inducing different degrees of intracellular acidification with nigericin (2.7 μM), and measuring the $\Delta p\text{H}_i$ resulting from the addition of NH_4Cl (2 mM). β_i was thus calculated as $\Delta[\text{NH}_4^+]/\Delta p\text{H}_i$ as

described [26,28], using a pK_a value of 9.21 for NH_4^+ . Linear regression analysis was then performed to determine the dependence of β_i on the initial pH_i .

Statistical analysis

Unless otherwise indicated, comparisons between two sample means were made by unpaired Student's *t* tests with analysis of variance.

RESULTS

The effect of polyamine depletion on pH_i was first assessed by measuring the equilibrium distribution of the weak acid [¹⁴C]DMOD in L1210 cells preincubated for up to 72 h with 5 mM DFMO. This ODC inhibitor leads to a virtually complete depletion of putrescine and spermidine between 24 and 48 h in these cells [22,30]. As shown in Table 1, a 3 day treatment with DFMO resulted in a fall in pH_i as measured in a HCO_3^- -free, Hepes-based buffer, and an approx. 70 % decrease in net cell growth. The change in pH_i observed did not always reach statistical significance, perhaps due to a greater inaccuracy of the weak acid distribution method (see below). Increasing the seeding density up to 3-fold did not significantly alter the effect of DFMO on pH_i , even when similar final densities were reached. Likewise, diluting control cell suspensions by 50 % 24 h after seeding in order to yield final densities similar to those of DFMO-treated cultures had no influence on pH_i (results not shown). Thus the DFMO-induced fall in pH_i probably did not result from cell-cycle-associated differences due to growth arrest and decrease in cell density [31].

[¹⁴C]DMOD tends to accumulate into mitochondria due to the higher pH prevailing in that organelle [24,27], and this factor can artificially increase the pH_i determined by the weak acid distribution method. We thus used the fluorescent dye BCECF [27] as an alternative tool for pH_i determination that might be less prone to partition into mitochondria once the acetoxymethyl ester is hydrolysed in the cytosolic compartment [32]. In these experiments, the pH_i was determined in complete HCO_3^- -containing RPMI 1640 medium in order to include the contribution of HCO_3^- -dependent mechanisms to pH_i regulation [27]. Indeed, the control pH_i as measured with BCECF was consistently lower than that with [¹⁴C]DMOD, but a 3 day incubation with DFMO still decreased steady-state pH_i by 0.25–0.32 unit in L1210 cells (Table 2). A near-maximal effect of DFMO on steady-state pH_i was already observed between 24 and 48 h of incubation, i.e. at the time of maximal putrescine and spermidine depletion (results not shown).

Polyamine analogues such as BE-3-4-3 and its derivatives are cytotoxic to many cell types by virtue of their ability to displace endogenous polyamines from the intracellular compartment [19], to repress polyamine biosynthesis [21] and to massively induce spermidine/spermine *N*-acetyltransferase [33], while being poor biological substitutes for natural polyamines [34]. We thus compared the effects of prior treatment with BE-3-4-3 and DFMO on steady-state pH_i (Table 3). Both DFMO and BE-3-4-3 decreased steady-state pH_i by about 0.4 unit after 3 days, while inhibiting growth to a similar extent. The effect of DFMO was completely reversed by either putrescine or spermidine, but not by BE-3-4-3. Neither exogenous putrescine nor spermidine had any effect on pH_i . As expected [22], DFMO influenced neither growth nor pH_i in ODC-overproducing L1210 cell variants (D-R), whereas BE-3-4-3 led to stable acidification as in parental cells. Thus the effects of DFMO and BE-3-4-3 on pH_i probably resulted from putrescine and polyamine depletion by these agents.

Table 1 Effects of DFMO and seeding density on pH_i as determined with [^{14}C]DMOD

L1210 cells were seeded at the indicated density in 75 cm² flasks in standard growth medium, and harvested after 72 h for pH_i determination by the [^{14}C]DMOD method. Cell number and pH_i values are means $\pm$ S.D. for three individual cell cultures. *Significantly different from control ($P < 0.05$).

Treatment	$10^{-5} \times$ Initial density (ml ⁻¹)	$10^{-5} \times$ Final density (ml ⁻¹)	pH_i
Control	0.5	16.9 $\pm$ 0.5	7.61 $\pm$ 0.08
+ 5 mM DFMO	0.5	5.1 $\pm$ 0.3	7.51 $\pm$ 0.03
+ 5 mM DFMO	1.0	9.6 $\pm$ 0.4	7.47 $\pm$ 0.03*
+ 5 mM DFMO	1.5	18.2 $\pm$ 0.1	7.32 $\pm$ 0.10*

Table 2 Effects of DFMO and seeding density on pH_i as determined with BCECF

L1210 cells were seeded at the indicated density in 75 cm² flasks in standard growth medium, and harvested after 48 h for pH_i determination by the BCECF method. Cell number and pH_i values are means $\pm$ S.D. for three individual cell cultures. For each cell culture, the pH_i value was averaged from three sequential determinations. *Significantly different from control ($P < 0.01$).

Treatment	$10^{-5} \times$ Initial density (ml ⁻¹)	$10^{-5} \times$ Final density (ml ⁻¹)	pH_i
Control	1.0	12.7 $\pm$ 0.1	7.48 $\pm$ 0.01
+ 5 mM DFMO	1.0	4.9 $\pm$ 0.8	7.16 $\pm$ 0.03*
+ 5 mM DFMO	2.0	6.6 $\pm$ 0.1	7.23 $\pm$ 0.01*
+ 5 mM DFMO	3.0	14.4 $\pm$ 0.1	7.22 $\pm$ 0.01*

Table 3 Effects of DFMO and BE-3-4-3 on cell proliferation and steady-state pH_i , and reversal of DFMO-induced acidification and growth inhibition by co-incubation with putrescine, spermidine and BE-3-4-3

Parental or ODC-overproducing (D-R) L1210 cells were seeded at 5×10^4 cells/ml in standard growth medium containing 1 mM aminoguanidine, and grown for 72 h under the indicated treatments. pH_i and cell number were then immediately determined by the BCECF method. ND, not determined. *Significantly different from control cells ($P < 0.01$); † significantly different from DFMO-treated cells ($P < 0.01$); ‡ significantly different from BE-3-4-3-treated cells ($P < 0.01$).

Treatment	$10^{-6} \times$ Cell number/ml		pH_i	
	L1210	D-R	L1210	D-R
Control	1.46 $\pm$ 0.01	1.46 $\pm$ 0.14	7.35 $\pm$ 0.03	7.30 $\pm$ 0.03
+ 50 μ M Putrescine	1.47 $\pm$ 0.09	ND	7.38 $\pm$ 0.02	ND
+ 10 μ M Spermidine	1.45 $\pm$ 0.07	ND	7.36 $\pm$ 0.01	ND
+ 25 μ M BE-3-4-3	0.30 $\pm$ 0.04*	0.56 $\pm$ 0.05*	6.92 $\pm$ 0.04*	7.02 $\pm$ 0.03*
+ 5 mM DFMO	0.41 $\pm$ 0.08*	1.56 $\pm$ 0.04	6.90 $\pm$ 0.02*	7.32 $\pm$ 0.02
+ 5 mM DFMO + 50 μ M putrescine	1.41 $\pm$ 0.05†	ND	7.33 $\pm$ 0.05	ND
+ 5 mM DFMO + 10 μ M spermidine	1.54 $\pm$ 0.21†	ND	7.35 $\pm$ 0.04	ND
+ 5 mM DFMO + 25 μ M BE-3-4-3	0.28 $\pm$ 0.04*	0.23 $\pm$ 0.01*‡	6.90 $\pm$ 0.03*	7.19 $\pm$ 0.04*‡

The time courses of the reversal of DFMO-induced growth inhibition and intracellular acidification by putrescine and spermidine are depicted in Figure 1. Partial restoration of pH_i was already detectable 6 h after the addition of putrescine to DFMO-treated cells, and a complete reversal of the effect of DFMO on pH_i was observed after 10 h (Figure 1a). Spermidine was more rapidly effective than putrescine in reversing the effect of DFMO on pH_i , a complete restoration of pH_i being induced by the polyamine as early as 2 h after addition (Figure 1a), whereas reversal of DFMO growth inhibition was detected between 10 and 24 h, as with putrescine (Figure 1b). Thus spermidine appears to be a more proximal or more efficient effector of pH_i regulation than putrescine.

A lower steady-state pH_i in polyamine-depleted cells might have resulted from an increased production of metabolic acid. However, there was no difference in the rate of acidification of the extracellular medium using dense cell suspensions in a weakly buffered solution (results not shown), thus suggesting that stable

intracellular acidification was not due to a higher metabolic rate. Therefore, in order to assess the pH_i regulatory mechanism(s) mainly affected by polyamine depletion, we measured the ability of DFMO- and BE-3-4-3-treated cells to restore pH_i after an acid load imposed by nigericin, an ionophore which catalyses electro-neutral H^+/K^+ exchange [28]. As shown in Figure 2(a), DFMO- and BE-3-4-3-treated L1210 cells exhibited a lower rate of intracellular alkalinization following the addition of NaCl to cell suspensions subjected to prior cytosolic acidification induced by nigericin in a Na^+ - and HCO_3^- -free buffer. The decrease in the rate of pH_i recovery was more accentuated as the pH_i became more alkaline, i.e. as pH_i reached stabilization. Interestingly, the initial difference in steady-state pH_i observed in polyamine-depleted cells under these conditions (-0.173 ± 0.042 and -0.150 ± 0.029 unit for DFMO- and BE-3-4-3-treated cells respectively; means $\pm$ S.D., $n = 3$) persisted after recovery from acid stress, indicating that the set-point for the alkalinizing mechanism was stably depressed as a result of polyamine

Figure 1 Comparison of the ability of putrescine and spermidine to reverse DFMO-induced growth inhibition and intracellular acidification in L1210 cells

Prior to the time course shown, L1210 cells were seeded at 1×10^5 cells/ml in 25 cm² flasks and incubated for 48 h in the absence (○) or presence of 5 mM DFMO. Putrescine (Put; □) or spermidine (Spd; ■) (50 and 10 μM respectively) was then added at time zero, and cell number (a) and pH_i (b) were determined after the indicated periods. pH_i was estimated using BCECF as described in the Materials and methods section. Values represent the means $\pm$ S.D. for three flasks.

Figure 2 Effects of DFMO and BE-3-4-3 pretreatment on pH_i recovery from nigericin-induced acid loading

(a) L1210 cells were grown for 3 days in the presence of 5 mM DFMO or 25 μM BE-3-4-3 or in the absence of either drug (control), and then labelled with BCECF and transferred to HCO_3^- -free NMG buffer. After a 90 s equilibration period, 1.4 μM nigericin was added to induce K^+/H^+ exchange-mediated acidification. Nigericin activity was then buffered with 0.5% (w/v) BSA, and NaCl (75 mM) was added at the time indicated. (b) As in (a), except that cells were supplemented with 500 μM amiloride at time zero of the time course shown. BCECF fluorescence was monitored and pH_i calculated as described in the Materials and methods section. The fluorescence ratio tracings shown are representative of six similar experiments for each treatment.

depletion. Amiloride (500 μM) partly blocked alkalization in control as well as in drug-treated cells (Figure 2b), suggesting that the Na^+/H^+ antiporter was responsible for a major fraction of the observed pH_i recovery. Similar results were obtained when lower amounts of NaCl or a higher (1 mM) amiloride concentration were added, indicating that the residual alkalization observed was not due to incomplete inhibition of Na^+/H^+ antiporter activity. This Na^+ -independent pH_i recovery was insensitive to as much as 100 nM bafilomycin A₁, suggesting that it was not mediated by a plasma membrane vacuolar ATPase [35]. Moreover, the new, lower equilibrium pH_i reached in the

presence of amiloride after acid stress was inferior by about 0.09 pH unit in DFMO- or BE-3-4-3-treated cells relative to control cells (Figure 2). The latter results suggest that an amiloride-resistant, HCO_3^- -independent mechanism is active in pH_i regulation in L1210 cells, and is sensitive to polyamine depletion.

The pH_i -dependence of the rate of alkalization after an acid load was then determined after allowing nigericin to induce a predetermined level of acidification under Na^+ - and HCO_3^- -free conditions. The effects of DFMO and BE-3-4-3 on β_i , as well as the dependence of β_i on pH_i , were measured in parallel in order to estimate the actual rate of proton efflux. There was no

Figure 3 Effects of DFMO and BE-3-4-3 pretreatment on the initial rate of H^+ efflux during recovery from nigericin-induced acidification to various initial pH_i values

L1210 cells were incubated for 48 h with 5 mM DFMO or 25 μ M BE-3-4-3, or in the absence of either drug (control), and then subjected to nigericin-mediated acid loading under HCO_3^- -free conditions as described for Figure 2. BSA (0.5%, w/v) was added when the pH_i had reached the desired initial value, followed by the addition of 75 mM NaCl to stimulate Na^+/H^+ antiporter activity. The initial rate of H^+ efflux was determined from the tangent to the fluorescence tracing immediately after NaCl addition, and from the estimated β_i value prevailing at the respective initial pH_i , as described in the Materials and methods section. The linear regression curves shown are representative of two similar experiments.

influence on β_i of polyamine depletion by either DFMO or BE-3-4-3, and β_i was found to be inversely related to pH_i in a linear fashion, as reported in other cell types [27], from 50 ± 12 to 19 ± 5 $mmol \cdot l^{-1} \cdot pH_i$ unit between pH_i values of 6.60 and 7.40 respectively. As shown in Figure 3, the initial rate of H^+ efflux decreased at a similar rate with increasing pH_i under all experimental conditions, but was lower in DFMO- or BE-3-4-3-treated cells at a given pH_i . Therefore the predicted equilibrium set-point (i.e. the initial pH_i at which $dpH_i/dt = 0$) was lower by about 0.25 unit in polyamine-depleted cells, in good agreement with the differences in steady-state pH_i (cf. Figure 2).

The effect of polyamine depletion on pH_i regulation was further examined by measuring the recovery from acid load caused by a NH_4Cl pulse. In this method, the pH_i is first raised upon diffusion and intracellular protonation of NH_3 , and then abruptly decreased due to rapid outward diffusion of NH_3 upon removal of NH_4Cl from the medium, leaving an intracellular excess of H^+ [31]. As shown in Figure 4, there were clearly two components in the pH_i recovery in L1210 cells subjected to NH_4Cl -induced acidification. After resuspension in a Na^+ -free buffer, a first phase of alkalinization was observed, at a rate which was very similar in control, DFMO- and BE-3-4-3-treated cells (0.060, 0.070 and 0.068 pH unit/min respectively). Upon NaCl addition, the rate of increase in pH_i was initially enhanced to a similar degree under both experimental conditions. However, the alkalinization rate decreased more rapidly in drug-treated cells, so that the pH_i stabilized again at a lower value than in control cells, as observed with nigericin-induced acid loads.

In order to assess further the effects of DFMO and BE-3-4-3 on Na^+/H^+ antiporter activity, control and drug-treated L1210 cells were subjected to hyperosmotic shock, which potently activates the Na^+/H^+ exchanger [36–39]. Increasing the osmo-

Figure 4 Effects of DFMO and BE-3-4-3 pretreatment on pH_i recovery from an NH_4Cl -induced acid load

Prior to the time course shown, L1210 cells were incubated for 48 h with 5 mM DFMO or 25 μ M BE-3-4-3, or in the absence of either drug (control), loaded with BCECF and transferred to 2 ml of Na^+ buffer at time zero with continuous recording of the I_{505}/I_{455} fluorescence ratio. NH_4Cl (20 mM) was added at the time indicated by further dilution of the cell suspension with 2 ml of Na^+/NH_4^+ buffer. Then, 2 min after the maximal pH_i was reached, cells were rapidly collected by centrifugation and resuspended in 2 ml of NMG buffer. NaCl (141 mM) was then added at the time indicated to stimulate Na^+/H^+ antiporter activity. The fluorescence ratio tracings are representative of three identical experiments.

lality by the addition of sucrose (250 mM) or an osmotically equivalent amount of NaCl (138 mM) rapidly raised the pH_i by 0.22–0.31 unit at a rate of 0.12 ± 0.02 pH unit/min in control L1210 cells (Figure 5a). Cellular alkalinization induced by hypertonic shock was strictly Na^+ -dependent, as shown by using sucrose as an osmoticum in a Na^+ -free buffer. Moreover, amiloride completely prevented the rise in pH_i following hypertonic shock induced by NaCl, strongly indicating that acid extrusion was exclusively mediated by the Na^+/H^+ exchanger. At the maximal pH_i reached in several hyperosmotic shock experiments (7.70 ± 0.02 , $n = 5$), subsequent addition of amiloride did not affect pH_i , suggesting that the antiporter had reached a new set-point at this pH_i value [36,38,40]. Osmotically induced alkalinization was reversible, as shown by the slow decrease in pH_i induced by restoring the initial osmolality (Figure 5a). On the other hand, hypertonic shock was equally effective in increasing the pH_i in DFMO- and BE-3-4-3-treated cells (final $pH_i = 7.75 \pm 0.06$ and 7.72 ± 0.05 respectively, $n = 4$), despite the 0.12–0.15 lower initial pH_i measured in these cells (Figure 5b). Likewise, the rate of alkalinization was not notably affected by either polyamine depletion strategy (0.16 ± 0.03 and 0.15 ± 0.02 pH unit/s for DFMO- and BE-3-4-3-treated cells respectively). Therefore Na^+/H^+ antiporter activity was still fully responsive to hypertonic shock after treatment with either DFMO or BE-3-4-3, suggesting that polyamine depletion does not decrease the total exchanger capacity.

Figure 5 Effects of DFMO and BE-3-4-3 pretreatment on intracellular alkalization induced by hyperosmotic shock

(a) Prior to the time course shown, L1210 cells grown for 48 h in standard growth medium were loaded with BCECF, and resuspended in either NaCl or NMG buffer, in the presence of 500 μ M amiloride where indicated. NaCl or sucrose (137 and 250 mM final concentrations in NMG and NaCl buffers respectively) was then added at the time shown. After equilibration to a maximal pH_i value, osmolality was restored to its initial value by diluting the suspension with Tris buffer. (b) L1210 cells were incubated for 48 h with 5 mM DFMO or 25 μ M BE-3-4-3, or in the absence of either drug (control), loaded with BCECF, and resuspended with 1.5 ml of NMG buffer. Cells were then subjected to hyperosmotic shock by addition of 137 mM NaCl in NMG buffer. Other details are as given in the Materials and methods section. The fluorescence ratio tracings shown are representative of three identical experiments.

DISCUSSION

The present data provide the first demonstration that polyamine depletion brought about either by ODC inhibition or by a dysfunctional polyamine analogue leads to stable intracellular acidification due to a persistent defect in pH_i regulation. This steady-state decrease in pH_i was not influenced by the growth status or density of the cell cultures. Furthermore, restoration of pH_i in DFMO-treated cells by exogenous putrescine and especially spermidine was rapid (from 2 to 8 h), and preceded the reversal of growth inhibition. These data suggest that polyamines exert a more direct action on pH_i -regulatory membrane activities than as mere permissive factors for rapid growth.

A stable decrease in pH_i at constant extracellular pH could arise in principle from any combination of several factors, including: (a) a decreased intracellular buffering capacity, (b) a higher rate of metabolic acid production, (c) an increased rate of proton influx, and (d) a decreased rate of active acid extrusion. Since polyamines are indeed strong bases, the overall rate of polyamine biosynthesis might impose a constant alkaline load and thus contribute to the intracellular buffering capacity. However, no evidence for a change in buffering capacity was obtained in the range of steady-state pH_i values found in control and polyamine-depleted cells. Moreover, the rate of extracellular acidification by cells suspended in a lightly buffered medium at normal extracellular pH (pH 7.40) was not affected by polyamine depletion caused by either DFMO or BE-3-4-3. Thus the decrease in steady-state pH_i in polyamine-depleted L1210 cells most likely

arose from changes in active alkalization mechanism(s) or increased proton influx.

A comparable decrease in pH_i was maintained in DFMO- or BE-3-4-3-treated cells kept in the absence or presence of HCO_3^- , thus indicating that HCO_3^-/Cl^- exchange mechanisms [41–45] could not correct the defect in pH_i regulation. On the other hand, a major effect of polyamine starvation was detected in the pH_i -dependence of the mechanisms responsible for intracellular alkalization after an acid load. This effect took the form of a decrease in the apparent set-point for the inactivation of active Na^+ -dependent pH_i regulation. From its sensitivity to amiloride and its lack of HCO_3^- -dependence, the major Na^+ -dependent mechanism of pH_i recovery from acid stress in L1210 cells was clearly the Na^+/H^+ antiporter activity. Thus one possible effect of polyamine depletion might be a stable lowering of the set-point of the Na^+/H^+ exchanger. An elevation of this set-point is an important mechanism of regulation of its activity by various agents such as phorbol esters, growth factors and hyperosmotic stress (reviewed in [38]). For instance polyamines, which have been reported to influence the phosphorylation of specific proteins [46,47], might be required for the phosphorylation of the Na^+/H^+ exchanger induced by growth factors, which has been correlated with the elevation of the pH_i set-point for antiporter activity [48]. Although ATP depletion is known to decrease the Na^+/H^+ antiporter affinity for intracellular H^+ [36,49,50], this mechanism is unlikely to be involved in the DFMO-induced stable intracellular acidification observed here, since polyamine depletion by DFMO in fact increases the ATP pool [51,52]. On

the other hand, the extent of stimulation of Na^+/H^+ antiporter activity observed after a hypertonic shock was unaffected by treatment with DFMO or BE-3-4-3, suggesting that the total Na^+/H^+ exchange capacity was not decreased by polyamine depletion. This would not rule out a durable effect of polyamine depletion on the Na^+/H^+ antiporter pH_i set-point since, unlike growth factors or phorbol esters, hyperosmotic shock does not modify the state of phosphorylation of the exchanger [53].

In addition to a Na^+/H^+ antiporter-like mechanism, a partial recovery from acid stress in L1210 cells was mediated by a less important, apparently Na^+ - and HCO_3^- -independent process (Figures 2 and 4). Since bafilomycin A_1 , a potent and specific inhibitor of vacuolar ATPases [35], did not affect that recovery, it cannot be ascribed to this class of proton extruding pumps, which has been found in the plasma membrane of various cell types [35,54]. Other Na^+ - and HCO_3^- -independent pH_i regulatory mechanisms have been described, such as a potential-dependent H^+ conductance [55], and the identity of this minor mechanism of pH_i recovery in L1210 cells remains to be determined.

Although cellular alkalinization by Na^+/H^+ antiporter activity has previously been proposed to participate in the early signalling mechanisms of mitogens, it is now more generally accepted that intracellular pH_i is rather stable at physiological pCO_2 through the additional contribution of Na^+ -dependent and -independent anion exchangers [41–45]. A stringent control of pH_i is indeed consistent with the pivotal role of this parameter in all aspects of cellular physiology. Nevertheless, a few pathological situations involve stable decreases in steady-state pH_i at physiological $\text{CO}_2/\text{HCO}_3^-$ concentrations, such as in lymphocytes from spontaneously hypertensive rats [56]. Moreover, a lower pH_i in tumour cells is linked to their thermosensitization [57]. Thus our current finding that the steady-state pH_i is stably decreased after treatment with DFMO or a polyamine analogue in a manner rapidly reversible by spermidine, and to a lesser degree putrescine, raises interesting questions as to the role of these compounds in pH_i regulation, the exact mechanism(s) responsible for the defect observed, and its involvement in the growth-inhibitory effect of polyamine depletion. It is expected that sustained intracellular acidification due to polyamine depletion should have far-ranging effects on almost all aspects of cell physiology, including intracellular Ca^{2+} /calmodulin-dependent processes and protein synthesis [36,41,58].

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