Hydrogen ion dynamics in human red blood cells

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Our understanding of pH regulation within red blood cells (RBCs) has been inferred mainly from indirect experiments rather than from in situ measurements of intracellular pH (pHᵢ). The present work shows that carboxy-SNARF-1, a pH fluorophore, when used with confocal imaging or flow cytometry, reliably reports pHᵢ in individual, human RBCs, provided intracellular fluorescence is calibrated using a ‘null-point’ procedure. Mean pHᵢ was 7.25 in CO₂/HCO₃⁻-buffered medium and 7.15 in Hepes-buffered medium, and varied linearly with extracellular pH (slope of 0.77). Intrinsic (non-CO₂/HCO₃⁻-dependent) buffering power, estimated in the intact cell (85 mmol (l cell)⁻¹ (pH unit)⁻¹ at resting pHᵢ), was somewhat higher than previous estimates from cell lysates (50–70 mmol (l cell)⁻¹ (pH unit)⁻¹). Acute displacement of pHᵢ (superfusion of weak acids/bases) triggered rapid pHᵢ recovery. This was mediated via membrane Cl⁻/HCO₃⁻ exchange (the AE1 gene product), irrespective of whether recovery was from an intracellular acid or base load, and with no evident contribution from other transporters such as Na⁺/H⁺ exchange. H⁺-equivalent flux through AE1 was a linear function of [H⁺]ᵢ and reversed at resting pHᵢ, indicating that its activity is not allosterically regulated by pHᵢ, in contrast to other AE isoforms. By simultaneously monitoring pHᵢ and markers of cell volume, a functional link between membrane ion transport, volume and pHᵢ was demonstrated. RBC pHᵢ is therefore tightly regulated via AE1 activity, but modulated during changes of cell volume. A comparable volume–pHᵢ link may also be important in other cell types expressing anion exchangers. Direct measurement of pHᵢ should be useful in future investigations of RBC physiology and pathology.

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Abbreviations CA, carbonic anhydrase; RBC, red blood cell.

Introduction

Red blood cells (RBCs) play a fundamental role in regulating the acid–base balance of extracellular fluids. Two key molecular elements for this in the RBC are haemoglobin (Hb) and the AE1 isoform of the anion-exchange transporter (also referred to as Band 3). Haemoglobin, at a concentration of 7 mmol per litre of cell water (mmol (l cell water)⁻¹), is the RBC’s main proton (H⁺ ion) buffer (Cass & Dalmark, 1973). AE1, expressed with over 10⁶ copies per human RBC (Alper, 1991), mediates a rapid transmembrane exchange of Cl⁻ for HCO₃⁻, thereby enhancing the mass transport of CO₂ in the form of plasma HCO₃⁻. AE1 activity is facilitated by cytoplasmic carbonic anhydrase (CA), an enzyme that catalyses the reversible hydration of carbon dioxide (CO₂ + H₂O ⇌ HCO₃⁻ + H⁺) (Meldrum & Roughton, 1933; Maren, 1967). Efficient AE1 activity in the RBC is critical for lung clearance of metabolically produced CO₂, and thus for the maintenance of plasma and whole-body pH.

While the role of RBCs in the regulation of plasma pH has been studied extensively (Van Slyke et al. 1923; Funder & Wieth, 1966), much less is known about hydrogen ion dynamics within the RBCs themselves. The control of RBC pHᵢ has usually been inferred from pH measurements after cell lysis (Cass & Dalmark, 1973), from changes of extracellular pH (pHₑ) during RBC activity (Freeman et al. 1987), from radiotracer measurements of Cl⁻ flux (Funder & Wieth, 1976), and from mathematical model predictions (Lew & Bookchin, 1986; Lew et al. 1991). Direct pHᵢ recordings, however,
have rarely been made. Progress in the field has been hindered by difficulties associated with monitoring intracellular ion concentrations in the intact RBC. The cells are too fragile and small to support stable impalement by ion-sensitive micropipettes. Furthermore, the use of some types of intracellular ion-sensitive fluorophore is frustrated by fluorescence quenching from intracellular Hb (Lew et al. 1993). A previous report has described the use of the pH fluorophore, BCECF, for recording average pH\textsubscript{i} in a population of RBCs (several million) suspended in solution within a cuvette (Kummerow et al. 2000). The method, however, was not validated for pH\textsubscript{i} measurement in individual cells, and the extracellular milieu was not controlled by continuous superfusion. In addition, BCECF has been shown to inhibit the RBC Ca\textsuperscript{2+}-ATPase (Gatto & Milanick, 1993), which may have a secondary impact on pH\textsubscript{i} and volume homeostasis.

In the present work, the potential problems of directly recording RBC pH\textsubscript{i} have been overcome with the use of the ratiometric pH indicator SNARF-1 (carboxy-seminaphthorhodafluor-1), diffused as the acetoxyethyl (AM) ester into the cell (Buckler & Vaughan-Jones, 1990). The dye appears not to affect RBC Ca\textsuperscript{2+}-ATPase activity, and it provides a signal sufficient for recording pH\textsubscript{i} in individual cells, despite the presence of Hb. Although difficulties were encountered with calibrating the signal using a classical ‘nigericin’ method (as also reported for renal mesangial cells; Boyarsky et al. 1996), an alternative, null-point method proved effective. Measurement of intracellular SNARF-1 fluorescence was combined with confocal imaging or flow cytometry, in order to characterise the control of pH\textsubscript{i} in the RBC, and its sensitivity to pH\textsubscript{o}. The work highlights major roles for intracellular Hb and plasmalemmal AE1. Of particular interest is pH\textsubscript{i} regulation in the presence of physiological levels of CO\textsubscript{2}/HCO\textsubscript{3}\textsuperscript{-}, as the majority of previous work on H\textsuperscript{+}-equivalent transport in the RBC has been performed in the nominal absence of this physiological buffer.

In addition to investigating pH\textsubscript{i} regulation, its role during changes of cell volume has also been explored. Direct measurements of pH\textsubscript{i} have been used to test key predictions of a mathematical model of RBC volume homeostasis (Lew & Bookchin, 1986; Lew et al. 1991). The model predicts that changes of pH\textsubscript{i} will modulate cell volume and pH\textsubscript{i} by influencing the activity of AE1. Similarly, RBC dehydration, induced by the opening of Ca\textsuperscript{2+}-activated KCNN4 (KCa3.1) channels in the plasmalemma, is predicted to cause a pH\textsubscript{i} change, again through recruitment of AE1 activity. This latter volume–pH\textsubscript{i} link has been suggested to occur in clinical episodes of sickle-cell anaemia (Bookchin et al. 1991; Lew & Bookchin, 2005), an inherited disease associated with the polymerisation of mutant haemoglobin (Hb S), leading to RBC dehydration. Activity of AE1 in the RBC is thus closely integrated into the mechanism of cell volume homeostasis. In the present work, simultaneous pH\textsubscript{i} and volume measurements have been made for individual RBCs, using confocal imaging and flow cytometry. Volume–pH\textsubscript{i} coupling has been assessed by manipulating pH\textsubscript{o}, or activating KCNN4 membrane channels (Gardos, 1958; Vandorpe et al. 1998; Begenisich et al. 2004). The results provide the first direct validation of pH\textsubscript{i} signalling during RBC volume regulation, and emphasise a key role for AE1.

Methods

Solutions and chemicals

The main solutions used were (in mM): ‘solution A’: NaCl, 142; KCl, 3; Hepes, 10; and MgCl\textsubscript{2}, 0.15; ‘solution B’: NaCl, 132; NaSCN, 10; KCl, 3; Hepes, 10; and MgCl\textsubscript{2}, 0.15; ‘solution C’: NaCl, 42; NaSCN, 10; KCl, 90; Hepes, 10; and MgCl\textsubscript{2}, 0.15. For superfusion experiments, solution pH was adjusted to 7.4 at 37°C. For flow cytometry experiments, solution pH was adjusted to 7.4 (unless stated otherwise) at 25°C. Some superfusion experiments were performed in the presence of physiological CO\textsubscript{2}/HCO\textsubscript{3}\textsuperscript{-} buffer, instead of Hepes. For these, solutions were modified to include 22 mM NaHCO\textsubscript{3} (replacing 22 mM NaCl) and bubbled with 5% CO\textsubscript{2} (balanced with air) at 37°C to equilibrate at a pH of 7.4. All solutions were in equilibrium with atmospheric O\textsubscript{2} partial pressure.

Where noted, additions were as follows (final concentrations in the cell suspension or in superfusate, in mM): NaOH-neutralized EGTA, 0.1; CaCl\textsubscript{2}, 0.25; NH\textsubscript{4}Cl, 30 (replacing 30 mM NaCl); sodium acetate, 80 (replacing 80 mM NaCl); sodium gluconate, 100 (replacing 100 mM NaCl); inosine, 5; pyruvate, 5; ionomycin (from 10 mM stock in DMSO), 0.01; acetoxymethyl (AM) ester of SNARF-1 (SNARF-1-AM), 0.01; nigericin, 0.01 μM; 4,4′-disothiocyanato-stilbene-2,2′-disulfonate (DIDS), 0.3 (Cabanchik & Rothstein, 1972).

SCN\textsuperscript{-} was used to by-pass the rate-limiting effects of anion permeation on the speed of dehydration of RBCs with upregulated K\textsuperscript{+} conductance. Ten millimolar SCN\textsuperscript{-} was sufficient to saturate this effect (Garcia-Sancho & Lew, 1988). Inosine was used as glycolytic substrate and pyruvate was added to bypass the glycolytic block imposed by the release of formaldehyde during the de-esterification of SNARF-1-AM (Tiffert et al. 1984; Garcia-Sancho, 1985). All chemicals were analytical reagent quality. NaSCN, sodium gluconate, EGTA, Hepes, DMSO, inosine, pyruvate, NS309, nigericin and ionomycin were from Sigma-Aldrich (Poole, UK). SNARF-1-AM was from Invitrogen (Paisley, UK). CaCl\textsubscript{2}, MgCl\textsubscript{2}, NaCl and KCl were from FSA Laboratory Supplies (Loughborough, UK).
Preparation of RBCs

After obtaining informed written consent, venous blood from healthy volunteers was drawn into tubes containing EDTA (ethylenediaminetetraacetic acid) to reduce plasma Ca\(^{2+}\) concentration and prevent clotting, washed twice in solution A with added EGTA, and twice more with solution A. The buffy coat (platelets and white cells) was removed after each wash. The washed RBCs were suspended at 0.1–1% haematocrit (Hct) in solution A, supplemented with inosine, pyruvate and SNARF-1-AM (10 μM) and incubated for 5 min at 37°C to allow for de-esterification and trapping of SNARF-1 inside RBCs.

RBC volume (measured using flow cytometry) followed for up to 40 min in solution A supplemented with 0.25 mM CaCl\(_2\), was not affected by SNARF-1 loading. This is relevant because if SNARF-1 had significant inhibitory effects on the plasma membrane Ca\(^{2+}\) pump, as claimed for the pH fluorophore BCECF (Gatto & Milanick, 1993), the cells would load with Ca\(^{2+}\), leading to dehydration and cell shrinkage (Lew & Bookchin, 1986). Figure S1 in the online Supplemental Material illustrates that exposure to 1 or 5 mM vanadate, a plasma membrane Ca\(^{2+}\) inhibitor, caused cell dehydration, while AM-loading to 10 μM vanadate, a plasma membrane Ca\(^{2+}\) pump inhibitor, caused cell dehydration, while AM-loading of SNARF-1 (10 μM) over the same time period (35 min) exerted no effect. SNARF-1 thus appears to exert little or no inhibitory effect on RBC Ca\(^{2+}\) homeostasis.

Superfusion and confocal fluorescence imaging

Superfusion was performed in a 2 ml Perspex chamber with a coverslip base, mounted on an inverted Leica IRBE microscope. A volume of 150 μl of the suspension with SNARF-1 loaded cells was carefully pipetted onto the coverslip, and the cells left to settle for about 10 min at room temperature. Cells were subsequently superfused with solution at 37°C, delivered at 2 ml min\(^{-1}\), to wash away extracellular SNARF-1 and any cells that had not settled on the coverslip surface.

Intracellular SNARF-1 was imaged confocally using a Leica TCS NT system. An argon laser excited the dye at 514 nm and fluorescence emission was collected at 580 nm and 640 nm simultaneously (with 40 nm band-pass filters) in xy scanning mode at a pixel resolution of 256 × 256 once every 1.1 s. To maximise emission signal, the pinhole was set to 2 Airy units. Fluorescence intensity at the two wavelengths was ratioed and converted to pH using a calibration curve.

Flow cytometry

A minimum of 150 μl of suspension with SNARF-1 loaded cells was aspirated through a flow cytometer (Quanta SC, Beckman Coulter, Brea, CA, USA). The intracellular dye was excited with a 488 nm laser and emission was collected at 630 nm and 575 nm through 30 nm band-pass filters. The ratio of fluorescence intensities at the two wavelengths was converted to pH using a calibration curve. Cell volume was measured simultaneously and independently of fluorescence. Volume calibration was performed using 10 μm-diameter spheres (Beckman Coulter). Due to limitations imposed by the equipment, measurements were performed at room temperature (25°C).

Experimental protocols

**In situ calibration of the SNARF-1 fluorescence ratio using nigericin.** Cells were superfused at 37°C (for confocal imaging) or suspended at 25°C (for flow cytometry) with high-K\(^+\) medium adjusted to different levels of pH within the range 5.5 to 9.5 by addition of HCl or NaOH from 5 M stocks. Two sets of calibration solutions were used for the 'nigericin method'. The first set (140-K) contained (in mM): NaCl, 5; KCl, 140; MgCl\(_2\), 0.15; Hepes (for the pH range 6.6–8.1) or Mes (for pH 5.5–6.6) or Hepps (for pH 8.1–9.5), 10. The second set (100-K) was a modification of the above, containing 100 mM KCl and 45 mM NaCl. All solutions also contained the K\(^+/H^+\) ionophore nigericin (10 μM). After allowing at least 2 min for equilibration, the SNARF-1 fluorescence ratio was plotted against solution pH to obtain a calibration curve (Thomas et al. 1979). The accuracy of the nigericin method relies on careful matching of intracellular K\(^+\) with extracellular K\(^+\) (Boyarsky et al. 1996), and therefore two sets of calibration solutions of different [K\(^+\)] were used to encompass the possible range of [K\(^+\)] inside RBCs.

**In situ calibration of the SNARF-1 fluorescence ratio using the null-point technique.** RBCs under confocal imaging were superfused with isosmotic solutions at 37°C containing a mixture of salts of a weak base (ammonium chloride) and weak acid (sodium acetate). For flow cytometry, cells were suspended at 25°C in similar solutions for 2–5 min before pH\(_i\) measurements were made. The [NH\(_4^+\)]/[acetate] ratio was varied in the different solutions (see Appendix for equations relating to the null-point method). At the 'null-point pH' (pH\(_{null}\)), exposure of an RBC to a particular ratio will produce no overall pH\(_i\) change, as the deprotonation of acetic acid within the cell is offset by the protonation of intracellular NH\(_3^+\) (Eisner et al. 1989; Buckler & Vaughan-Jones, 1990). The null-point solutions at 37°C had the following concentrations of NH\(_4\)Cl and sodium acetate (in mM): 'pH 7.5': 5, 3.08; 'pH 7.4': 5, 4.9; 'pH 7.3': 5, 7.75; 'pH 7.2': 5, 12.28; 'pH 7.1': 5, 19.45; 'pH 7.0': 2, 12.33; 'pH 6.9': 2, 19.55; 'pH 6.8': 2, 31; 'pH 6.7': 2, 49.1. The null-point solutions at 25°C had the following concentrations of NH\(_4\)Cl and sodium acetate (in mM): 'pH 7.3': 5, 7.83; 'pH 7.2': 5, 12.41; 'pH 7.1': 5, 19.67; 'pH 7.0': 5, 31.18. These
calculations have assumed a pK for NH$_4^+$ and acetic acid of 9.03 and 4.528 at 37°C, and 9.25 and 4.75 at 25°C. The null-point method assumes that the weak acid/base enters cells principally in the uncharged form. To block any possible entry of acetate or ammonium ions via anion exchange and K$^+$ channels, respectively, all null-point solutions contained DIDS (300 μM) and Ba$^{2+}$ (1 mM) (Leem et al. 1999). The Appendix provides further details of the assumptions and equations used in the null-point technique.

**Measurement of RBC hydrogen ion buffering capacity.** Individual RBCs were superfused with solution A in the presence of DIDS (300 μM), followed by solutions containing NH$_4$Cl (30, 15, 5, 0 mM; replacing NaCl isotonically) for at least 2 min each. The fall of pH$_i$ (ΔpH$_i$) on [NH$_4^+$]$_o$ reduction was imaged confocally, and the Henderson–Hasselbalch equation was used to estimate the fall of intracellular [NH$_4^+$] (equal to the concentration of H$^+$ ions released into the cell, C$_{H^+}$), assuming that the pK of NH$_4$Cl in cytoplasm is the same as in the extracellular solution. Intracellular buffering power (β) was estimated as: β = −ΔC$_{H^+}$/ΔpH$_i$ = Δ[NH$_4^+$]/ΔpH$_i$ (Boyarsky et al. 1988). This value was referenced to the midpoint of the fall of pH$_i$ on [NH$_4^+$]$_o$, reduction.

**Tests of model predictions.** Our current understanding of red blood cell homeostasis is encoded in the mathematical model of Lew & Bookchin (1986). The model includes a non-allosteric formulation for AE1, represented as an model of Lew & Bookchin (1986). The model includes membrane-permeant weak acid and base (sodium acetate and ammonium chloride) were added simultaneously to the extracellular superfusate (at a pH$_o$ of 7.4), and a combination of concentrations was found that exerted the smallest effect on steady-state pH$_i$. This ‘null’ combination allows one to deduce the value of steady-state pH$_i$. The technique does not require nigericin and therefore does not rely on accurately matching [K$^+$]$_o$, 0.2 units more alkaline than previous indirect estimates of pH$_i$, and is higher than predictions based on the transmembrane [Cl$^-$] gradient ([Cl$^-$]$_o$, > [Cl$^-$]), therefore [H$^+$]$_i$, > [H$^+$]$_o$, ; Jacobs & Stewart, 1947; Salenius, 1957; Salminen & Manninen, 1966; Dalmark, 1975), suggesting a nigericin-based calibration error. The calibration was therefore crossed-checked using a ‘null-point’ technique (Eisner et al. 1989; Boyarsky et al. 1996).

Salt of a membrane-permeant weak acid and base (sodium acetate and ammonium chloride) were added simultaneously to the extracellular superfusate (at a pH$_o$ of 7.4) and a combination of concentrations was found that exerted the smallest effect on steady-state pH$_i$. This ‘null’ combination allows one to deduce the value of steady-state pH$_i$. The technique does not require nigericin and therefore does not rely on accurately matching [K$^+$]$_o$, 0.2 units more alkaline than previous indirect estimates of pH$_i$, and is higher than predictions based on the transmembrane [Cl$^-$] gradient ([Cl$^-$]$_o$, > [Cl$^-$]), therefore [H$^+$]$_i$, > [H$^+$]$_o$, ; Jacobs & Stewart, 1947; Salenius, 1957; Salminen & Manninen, 1966; Dalmark, 1975), suggesting a nigericin-based calibration error. The calibration was therefore crossed-checked using a ‘null-point’ technique (Eisner et al. 1989; Boyarsky et al. 1996).

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**Results**

**Calibration of intracellular SNARF-1 fluorescence**

Individual RBCs were superfused with high-K$^+$ (140 mM) nigericin-containing solutions adjusted to a range of pH values (Thomas et al. 1979). As shown in the left panel of Fig. 1A, the intracellular SNARF-1 emission ratio responded dynamically to changes in pH$_o$. This method of in situ dye calibration relies on the capacity of nigericin to equalise pH$_i$ with pH$_o$, provided [K$^+$]$_o$ is set to equal [K$^+$]$_i$. As [K$^+$], is known to fall with RBC age (Lew et al. 2007), the calibration procedure was repeated with RBCs superfused with 100 mM [K$^+$]$_o$ media (right hand panel of Fig. 1A), in order to bracket the likely [K$^+$]$_i$, range (100–140 mM). The averaged pH sensitivity of the fluorescence ratio is plotted in Fig. 1C for both 100 mM (filled grey circles) and 140 mM [K$^+$]$_o$ (open circles). The two sets of results are virtually identical.

The calibrations shown in Fig. 1A suggest that resting RBC pH$_i$ was about 7.4 (ratio = 0.8), similar to the pH of normal Hepes-buffered superfuse. This value is about 0.2 units more alkaline than previous indirect estimates of pH$_i$, and is higher than predictions based on the transmembrane [Cl$^-$] gradient ([Cl$^-$]$_o$, > [Cl$^-$]), therefore [H$^+$]$_i$, > [H$^+$]$_o$, ; Jacobs & Stewart, 1947; Salenius, 1957; Salminen & Manninen, 1966; Dalmark, 1975), suggesting a nigericin-based calibration error. The calibration was therefore crossed-checked using a ‘null-point’ technique (Eisner et al. 1989; Boyarsky et al. 1996).

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**Figure 1Ba** shows a sample time course for a null-point determination, performed on an RBC superfused with nominally CO$_2$/HCO$_3^-$-free solution (Hepes buffered). The transient alkaline displacements of pH$_i$, are due to the higher membrane permeability of ammonia, compared to acetic acid. Steady-state pH$_i$, is, however, determined by the equilibrium concentration of weak acid and base, and independent of the relationship between ammonia and acetic acid permeability constants (see Appendix for the derivation). The weak acid/base combination for a pH$_{null}$ of 7.1 produced the smallest steady-state displacement of the SNARF-1 emission ratio, indicating that resting pH$_i$, was near 7.1, close to the expected value for RBCs. This is quantified further in Fig. 1Bb, which plots the steady-state ratio displacement, for the four null-point solutions used. By interpolation, the pH$_i$, at which the ratio is expected to remain unchanged (the true null-point, equal to resting pH$_i$) was 7.13.
Best-fitting the nigericin calibration data (Grynkiewicz et al. 1985) indicated an apparent $pK_{SNARF}$ for the intracellular ratio of 7.26 (Fig. 1C). In contrast the null-point data, averaged for several cells, yielded an intracellular $pK_{SNARF}$ that was acid-shifted by 0.18 units (see inset to Fig. 1C). A typical resting RBC $pHi$, derived from the null-point method, will thus be significantly lower than that from the nigericin method. Moreover, the lower value (see next section) is consistent with previous, indirect estimates of $pHi$. A combined approach to in situ SNARF-1 calibration was also undertaken for RBC experiments using flow cytometry rather than confocal imaging (i.e. nigericin and null-point calibrations were compared). The results, shown in Supplemental Fig. S2, indicate a similar discrepancy of $\sim 0.2$ pH units between the two methods, again suggesting inadequacy in the nigericin method, as applied to RBCs.

In most other cell types where in situ nigericin and null-point calibrations of intracellular pH fluorophore signals have been compared, the resulting $pHi$ values have been virtually identical (see Discussion). This is also true for a range of cultured cell lines (gift from Prof Adrian L. Harris, Oxford, UK), as reported in Supplement Fig. S3. In the isolated renal mesangial cell, however, an error of up to 0.2 pH units, comparable to that seen in RBCs, has been reported for nigericin but not for null-point calibrations (Boyarsky et al. 1996). Because the nigericin-based $pHi$ estimates could not be reconciled with the well documented equality of $Cl^-$ and $H^+$ concentration ratios across RBC membranes, the null-point calibration was applied to estimate $pHi$ in all subsequent work reported in this paper.

**Resting intracellular pH and its dependence on extracellular pH**

Figure 2A shows the frequency distribution of resting $pHi$ (at $pHo$ 7.40), determined from confocal imaging of individual RBCs. In Heps-buffered medium, mean $pHi$ was 7.15, with a coefficient of variation (c.v.) of 1.01% (mean and c.v. determined from the best-fit normal distribution). In 5% CO$_2$/HCO$_3^-$ buffered medium, mean $pHi$ was 7.25 (c.v. = 0.99%), a slightly more alkaline value.
Resting $pHi$ in Hepes-buffered medium was analysed further by flow cytometry. SNARF-1 fluorescence was measured in RBCs pre-equilibrated in solutions of different pH (6.6, 7.05, 7.4 and 7.7 at 25°C). The frequency distribution of RBC $pHi$ in each of the four solutions was approximately normal (Fig. 2A). Mean steady-state $pHi$ is plotted as a function of $pHo$ in Fig. 2B. The relationship is linear, with a slope of 0.77 $pHi/pHo$ unit. A fall of $pHi$ thus induces a fall of $pHi$ of nearly the same magnitude. The dashed line (slope = +0.86 $pHi/pHo$) plots the RBC model prediction for the $pHi$–$pHo$ relationship, which is based upon the assumption that AE1 carries net $H^+$-equivalent fluxes until reaching equilibrium ($[Cl^-]_o/[Cl^-]_i = [H^+]_i/[H^+]_o$). The experimental measurements are in good agreement with the model predictions. The change of $pHi$ with $pHo$ is predicted, in the model, to be mediated by transmembrane $H^+$-equivalent flux through AE1. This was confirmed experimentally by applying the AE1 inhibitor DIDS before imposing $pHo$ changes, which eliminated the sensitivity of $pHi$ to changes of $pHo$ (Fig. 2Bb).

**Dynamic responses of intracellular pH to membrane-permeant weak acids and bases**

Superfusion of an individual RBC with NH$_4$Cl or sodium acetate generated, respectively, a rapid cell alkalinisation and acidification (Fig. 3A and B). The $pHi$ then recovered back towards control levels. Similarly, the subsequent
removal of these salts from the superfusate induced, respectively, an intracellular acid load and base load, again followed by a secondary pH$_i$ recovery. The pH$_i$ transients were qualitatively similar when recorded under Hepes- or CO$_2$/HCO$_3^-$-buffered conditions (Fig. 3Ab), although pH$_i$ recovery from acidosis was notably faster with the latter buffer.

The secondary pH$_i$ recovery from the alkalosis induced by ammonium addition (Fig. 3Aa), or by acetate removal (Fig. 3Cb), was largely abolished when the anion exchange inhibitor DIDS (300 μM) was added to the superfusate. This result indicates a major role for Cl$^-$/HCO$_3^-$ exchange in restoring pH$_i$ from acute base loading, as similarly reported in many types of eukaryotic cell (Vaughan-Jones, 1982; Aickin, 1994; Sun et al. 1996). The absence of a secondary acidification during exposure to ammonium-containing solution in the presence of DIDS also suggests that NH$_4^+$ entry into cells is minimal. The pH$_i$ recovery from acetate-induced intracellular acidosis (Fig. 3B), or from acidosis induced by ammonium-removal (Fig. 3Ca), was also blocked by DIDS, implying that AE1 is capable of restoring pH$_i$ from both an acid and an alkaline load. In contrast, pH$_i$ recovery from an acute acid or base load was unaffected by the Na$^+$/H$^+$ exchange (NHE) inhibitor dimethyl amiloride (DMA; Fig. 3Ca and b), suggesting no significant role for this H$^+$ transporter in RBC pH$_i$ regulation. When combined with previous findings that AE1 is the only known HCO$_3^-$ transporter expressed in RBCs, the present results suggest that pH$_i$ is tightly and symmetrically regulated by the anion exchanger.

Figure 3. Hydrogen ion dynamics during exposure to salts of weak acid or base
SNARF-1 loaded RBCs were imaged confocally and superfused at 37°C with solutions at pH 7.4. Time courses shown are averages of 5 cells. A, transient exposure of cells to solution containing 30 mM ammonium chloride (Am) under (a) 10 mM Hepes-buffered or (b) 5% CO$_2$/22 mM HCO$_3^-$-buffered conditions. Ammonium produced an immediate cell-alkalinisation followed by pH$_i$ recovery. On removal of ammonium, the cell acidified and then recovered towards resting pH$_i$. B, transient exposure of cells to solution containing 80 mM sodium acetate (Ac) under 10 mM Hepes-buffered conditions. Acetate produced an immediate cell-acidification followed by pH$_i$ recovery. On removal of acetate, the cell alkalinised and then recovered towards resting levels. Slow pH$_i$ recovery during acetate or ammonium exposure was blocked by the AE1 inhibitor DIDS (300 μM). C, recovery of pH$_i$ following (a) a 30 mM ammonium prepulse or (b) an 80 mM acetate prepulse was not slowed by the Na$^+$/H$^+$ inhibitor dimethylamiloride (DMA; 30 μM), but blocked by the AE inhibitor DIDS (300 μM). Drugs were added after ammonium or acetate removal in order to produce adequate acid or base loads for analyses. Averages of 20 cells; error bars omitted for clarity (S.E.M. values < 0.075 pH$_i$ units).
Intracellular and CO₂/HCO₃⁻-dependent intracellular buffering capacity

Intracellular H⁺-buffering capacity (β) was estimated in situ by titrating the cytoplasmic compartment with acid. This was achieved by a stepwise reduction in the concentration of ammonium ions in the superfusate. Figure 4A shows that this induced a stepwise fall of pHi, as intracellular [NH₄⁺] was successively depleted via NH₃ efflux, thus depositing intracellular H⁺ ions (Boyarsky et al. 1988). DIDS was included in all superfusates to block pHᵢ recovery through AE1. The pHᵢ steps were used to estimate β (see Methods). Figure 4B shows binned data for β (= −ΔCᵢH/ΔpHᵢ = Δ[NH₄⁺]/ΔpHᵢ), determined from experiments performed in the presence or absence of CO₂/HCO₃⁻ buffer. In nominally CO₂/HCO₃⁻-free, Hepes-buffered medium, there was a shallow decline of intrinsic β values over the pHᵢ range 7.0–7.35, from 100 to 75 mmol (l cell⁻¹) (pH unit)⁻¹ (equivalent to 130–100 mmol (l cell water⁻¹) (pH unit)⁻¹; assuming water occupies 75% of cell volume; Salenius, 1957; Salminen & Manninen, 1966). Under these conditions, the major intrinsic RBC buffer is haemoglobin, which is rich in H⁺-binding histidine residues, with pK values within the physiological pH range.

Intracellular β estimated in the presence of CO₂/HCO₃⁻ was higher than in Hepes buffered medium (Fig. 4B), and was essentially independent of pHᵢ. The increase in β is due to additional (extrinsic) buffering from intracellular CO₂/HCO₃⁻, and was numerically in agreement with that predicted from [HCO₃⁻], assuming CO₂ instantly equilibrates across the RBC membrane (Roos & Boron, 1981; Leem et al. 1999) (β = 2.303 × [HCO₃⁻]; = 2.303 × [HCO₃⁻]ₐ × 10⁻¹·ₙ·pHᵢ).

H⁺-equivalent flux through anion exchange and its dependence on intracellular pH

Using estimates of buffering capacity (Fig. 4) and the time courses of pHᵢ recovery from acid or base loads (Fig. 3C), it was possible to characterise the H⁺-equivalent flux (J_H) produced by AE1 in the presence or absence of CO₂/HCO₃⁻ buffer,

\[ J_H = -\frac{d \text{pH}}{dt} \times \beta \]

Measurements of dpH/dt were made 1 min after the removal of weak acid (acetate) or base (ammonium) to ensure that all intracellular ammonium/acetate has been vented out of cells. Figure 5 shows binned data for J_H, plotted as a function of pHᵢ (Fig. 5A) or intracellular [H⁺] (Fig. 5B). Flux data obtained in 5% CO₂/22 mM HCO₃⁻ or Hepes-buffered superfusates are plotted separately. Note that flux is boosted considerably (over fivefold) in the presence of CO₂/HCO₃⁻ buffer. The dependence of J_H on [H⁺], is roughly linear, with flux reversal occurring in the region of resting pHᵢ (72 nm [H⁺] in Hepes buffer, and approaching a reversal value of 65 nm [H⁺] in CO₂/HCO₃⁻ buffer). For data gathered in Hepes buffer, the exponent (n) for the best-fit by the function J_H = a + b × [H⁺]ⁿ was 0.73, which is close to an exponent of 1.0 (a and b are constants). The near-linearity of the H⁺ dependence of flux indicates that the AE1 transporter is not regulated co-operatively by intracellular H⁺.
ions. The data thus argue for a lack of allosteric control of the transporter by pH_i.

Given that AE1 does not transport H^+ ions directly, the J_H^-pH_i relationship shown in Fig. 5A most likely describes the substrate dependence of transmembrane H^+-equivalent flux. The H^+-equivalent substrate transported on AE1 is HCO_3^-, probably with no simultaneous transport of OH^- ions (Knauf et al. 2002). [HCO_3^-]_i is a function of pH_i and [CO_2]. Since [CO_2] was maintained constant during experiments, at either the atmospheric level of 0.04% (with Hepes-buffered superfusates), or at 5% (with CO_2/HCO_3^- buffered superfusates), J_H data were re-plotted and re-binned (Fig. 5C) as a function of [HCO_3^-]_i, normalised to [CO_2]:

\[
\frac{[\text{HCO}_3^-]}{[\text{CO}_2]} = \frac{K}{[\text{H}^+]} \]

where K is 10^{-6.15} M. This transformation allows the Hepes and CO_2/HCO_3^- data-sets to be plotted over a comparable x-axis range. The equilibrium condition (CO_2 + H_2O ⇌ HCO_3^- + H^+) that is necessary for this conversion is satisfied by the high intracellular CA activity and by cell superfusion with solutions pre-equilibrated with CO_2/HCO_3^-.

The linearity between J_H and the intracellular [HCO_3^-]/[CO_2] ratio confirms that, under conditions where [Cl^-]_o and [HCO_3^-]_o are constant, flux through AE is most likely instructed by [HCO_3^-]. Although not tested experimentally, deviations from linearity would be expected at high [HCO_3^-]_i due to saturation of binding to the transport site, and possibly at extremes of pH_i due to changes in [Cl^-], that arise from constraints set by the osmotic and electric stability of RBCs.

The time courses of pH_i recovery following an ammonium (Fig. 3Ca and 3Ab) or acetate (Fig. 3Cb) prepulse, in the absence of DIDS or DMA, are replotted in Fig. 5D. These are superimposed with the predicted pH_i time courses derived from the empirical J_H^-pH_i relationship (Fig. 5A). The good agreement between simulation and experimental data is again consistent with a lack of H^+ cooperativity and the ability of AE1 to produce acid or base extrusion depending on the prevailing transmembrane gradients for HCO_3^- and Cl^-.

Response of intracellular pH to changes in extracellular Cl^-}

The above results establish that pH_i is efficiently regulated by AE1 (Fig. 3) and can be reset by changing pH_o.
Resting pH$_i$ was also sensitive to changes of extracellular [Cl$^-$]. In the experiment shown in Fig. 6A, SNARF-1 loaded cells were superfused with an iso-osmotic low-Cl$^-$, Hepes-buffered solution (100 mM Cl$^-$ replaced by membrane-impermeant gluconate). This manoeuvre generated a reversible intracellular alkalosis that was fully inhibited by DIDS, indicating Cl$^-$ efflux in exchange for HCO$_3^-$ influx through AE1. The initial H$^+$-equivalent efflux into low Cl$^-$ was 15.5 ± 1.0 mM min$^{-1}$, while initial H$^+$-equivalent influx on restoration of Cl$^-$ was 24 ± 1.2 mM min$^{-1}$. This asymmetry of flux magnitude, which is consistent with the asymmetry of intracellular versus extracellular Cl$^-$ concentration ([Cl$^-$]$_i$ > [Cl$^-$]$_o$), agrees well with the predictions (Fig. 6B) of a model featuring AE activity that tends to restore equilibrium ([Cl$^-$]$_o$/[Cl$^-$]$_i$ = [H$^+$]$_i$/[H$^+$]$_o$), without allosteric restrictions imposed by H$^+$ ions. An alkaline-shift in the frequency distribution of resting pH$_i$ upon Cl$^-$ reduction, was also measured by flow cytometry after 10 min of gluconate exposure (at 25°C), as shown in the inset.

Extracellular Cl$^-$ reduction was repeated in the presence of CO$_2$/HCO$_3^-$ buffer (Fig. 6C). The reversible alkalisation was now much faster (probably rate-limited by the data acquisition rate), as expected from the higher concentration of AE1-substrate, HCO$_3^-$ (at pH$_o$ 7.4), and was not blocked completely by DIDS. During the rapid acid efflux associated with Cl$^-$ removal, pH$_i$ imaged confocally within individual RBCs remained spatially uniform to within 0.05 pH units (data not shown). This result suggests that cytoplasmic H$^+$ diffusion is sufficiently fast to maintain spatial pH$_i$ uniformity, even during the

![Figure 6. Cell-alkalinisation in response to removal of extracellular chloride](image)
large H+-equivalent transmembrane fluxes recorded in CO₂/HCO₃⁻ buffer.

**Coupling of RBC volume and pH**

Possible functional links between the control of RBC pHᵢ and volume were investigated. To do this, two manoeuvres that influence RBC volume were tested for effects on pHᵢ: a change of pH₀ and an increase in membrane K⁺ conductance.

As shown in Fig. 7Aa, steady-state RBC volume, measured at pH₀ 7.40 by flow cytometry, was normally distributed with a mean value of 89.8 fl and a C.V. of ~14%. This mean value decreased to 87.0 fl when pH₀ was raised to 7.7, and increased to 91.4 fl at pH₀ 7.05, and 93.8 fl at pH₀ 6.6. The final mean volumes (also normally distributed) are in agreement with previous measurements (Lew et al. 1995). A modest decrease of RBC volume thus results from a rise of pH₀ over the physiological range. For convenience, the inset reproduces the rise of pHᵢ that accompanies a rise of pH₀, as also shown in Fig. 2. It is notable that the changes of volume and pHᵢ were both inhibited by 300 μM DIDS (Fig. 7Ab), confirming that modulation of pHᵢ by pH₀, which is mediated via H⁺-equivalent flux through the AE1 transporter, was driving the volume changes. Figure 7B combines data from Fig. 7Ab and the inset into a single plot, emphasising that cell volume declines by 9% per unit rise of pHᵢ.

KCl efflux, when activated, also changed volume and pHᵢ. When fully activated, the Ca²⁺-sensitive K⁺ (Gardos) channel (KCNN4, otherwise known as KCa3.1) of human RBCs can mediate net K⁺ efflux that is two orders

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**Figure 7.** Cell volume is linked with pHᵢ

Aa, cell volume and pHᵢ were measured in RBCs bathed in iso-osmotic solutions at different pH₀ by flow cytometry at 25°C. b, cell volume increased in response to a fall in pH₀. This effect was blocked by 300 μM DIDS, implying a role for AE in the pH₀-volume link. DIDS also collapsed the pHᵢ–pH₀ link (inset). B, inverse relationship between pHᵢ and cell volume, with a slope of −7.9 fl/pHᵢ.
of magnitude higher than the normal resting K\(^+\) leak (Lew & Ferreira, 1978), and exceeds the restorative capacity of the Na\(^+\)/K\(^+\)-pump. AE1, operating in ion slippage or tunnelling mode, mediates an electrogenic Cl\(^-\) conductance that allows Cl\(^-\) efflux to follow the K\(^+\) current. Evidence for electro-diffusional Cl\(^-\) flux via AE1 has been derived from the similarity in the sensitivity of electrogenic Cl\(^-\) flux and Cl\(^-\)/HCO\(_3\)\(^-\) exchange to stilbene drugs (Kaplan et al. 1983; Knauf et al. 1983), and the reduced DIDS-sensitive Cl\(^-\) conductance in AE knock-out murine RBCs (Alper et al. 2008). The loss of water in response to KCl loss leads to RBC dehydration (see Supplemental Fig. S4 for a detailed schematic representation of the underlying flux). The decrease of [Cl\(^-\)], is then predicted to lower pH\(_i\) via AE1-mediated HCO\(_3\)\(^-\) efflux in exchange for Cl\(^-\) influx (Lew & Bookchin, 1986).

To test for a coupling between volume and pH\(_i\) during K\(^+\) channel opening, RBCs were superfused with solution B containing 250 \(\mu\)M Ca\(^{2+}\) and then exposed to either 1.5 \(\mu\)M ionomycin (a Ca\(^{2+}\) ionophore; Fig. 8Aa) or 10 \(\mu\)M NS309 (a Gardos channel activator; Fig. 8Ab). Intracellular pH and cell volume were recorded confocally from the SNARF-1 fluorescence signals. The pH\(_i\) was measured in the usual way, from the SNARF-1 ratio. The fluorescence signal was also used to visualise the outline of an RBC, permitting measurement of its mean diameter in the xy plane. Cell dehydration was assessed from the absolute increase in fluorescence intensity, interpolated to the dye’s isosbestic wavelength (620 nm). Fluorescence intensity and cell-diameter are only approximate indices of cell volume and therefore their temporal correlation with pH\(_i\) must be interpreted with caution. Figure 8A illustrates an experiment where the addition of Ca\(^{2+}\) ionophore triggered a prompt and progressive intracellular acidification of about 0.14 unit, together with a reduction in cell diameter and a delayed increase in SNARF-1 fluorescence intensity, both indicative of progressive cell dehydration. K\(^+\) channel opening thus decreased cell volume and pH\(_i\). The cause of the small initial fall in overall fluorescence intensity upon K\(^+\) channel opening, which preceded the fluorescence increase (Fig. 8Aa, lower panel), is not known. It may be due to a decrease in overall fluorescence yield during acidification. The apparently delayed response of cell diameter, relative to acidification, may reflect a change in volume along the z-axis that is not reflected in the xy plane. The sustained change in cell diameter and fluorescence intensity after pH\(_i\) had stabilised at a new acidic level may reflect changes in cell shape at constant volume (Cueff et al. 2010). Nevertheless, the combined measurement of cell diameter and intracellular fluorescence intensity provides a good, albeit semi-quantitative indication of cell dehydration.

NS309 is a well characterized positive modulator of Gardos channels (Strobaek et al. 2004; Baunbaek & Bennekou, 2008). It acts by increasing the Ca\(^{2+}\) sensitivity of the channels, such that they become capable of dehydrating RBCs at physiological or even sub-physiological [Ca\(^{2+}\)] \(<50\) nM (Baunbaek & Bennekou, 2008). The effect of NS309 on RBC volume and pH is shown in Fig. 8Ab. Again, cell dehydration, monitored confocally, was associated with an intracellular acidification of \(\sim0.1\) units. The pH\(_i\) and volume responses to ionomycin and NS309 were both abolished by blocking AE1 with DIDS (Fig. 8Bb and b), implying a role for AE1 in the volume–pH\(_i\) coupling.

Further experiments were performed with flow cytometry to obtain the steady-state relationship between pH\(_i\) and cell volume during K\(^+\) channel activation. Figure 8C plots population-averaged data, under control conditions and in the presence of NS309 or ionomycin, after allowing 15–30 min for the drugs to exert their effects. During K\(^+\) channel activation, a 0.1 unit fall of pH\(_i\) from its resting level (7.17) was associated with a 20% decline in cell volume. To augment the effect of NS309, one set of experiments was performed in the presence of the Ca\(^{2+}\) pump inhibitor, 1 mM vanadate, to raise intracellular [Ca\(^{2+}\)]. Vanadate potentiated the effect of NS309, producing a more dehydrated and more acidic population of RBCs (Fig. 8C). This finding also suggests that, under control conditions, the Ca\(^{2+}\) pump remains functional in the presence of intracellular SNARF-1, as suggested earlier (see also Supplemental Fig. S1).

In summary, direct measurements of RBC pH\(_i\) indicate that it changes significantly during manoeuvres that influence cell volume. The direction of pH\(_i\) change, however, depends on the underlying cause of the volume change. RBC shrinkage induced by extracellular alkalisation is associated with a rise of pH\(_i\), whereas shrinkage induced by K\(^+\) channel activation reduces pH\(_i\). In both cases, AE1 transport activity drives the pH\(_i\) change.

**Discussion**

The present work reports the first direct recordings of pH\(_i\) in individual RBCs. The measurements have permitted a characterisation of pH\(_i\) regulation in these cells, and its functional coupling to mechanisms that regulate cell volume. The results highlight the RBC’s exceptionally high intracellular buffering capacity, the unusual role of the AE1 gene product as both an acid and a base extruder following cytoplasmic acidosis or alkalosis, and the occurrence of AE1-driven pH\(_i\) signals during cell shrinkage or swelling. Intracellular pH is thus tightly regulated in the RBC, but modulated by the cell’s volume.
Resting pH_i in the human RBC

The mean resting pH_i of 7.25 (i.e. less than pH_o 7.4) is consistent with previous indirect estimates (Jacobs & Stewart, 1947; Salenius, 1957; Salminen & Manninen, 1966; Dalmark, 1975), the value becoming somewhat more acidic (7.15) in the nominal absence of physiological CO_2/HCO_3^- buffer. The more alkaline pH_i in the presence of CO_2/HCO_3^- buffer cannot be explained by differences

Figure 8. Cell dehydration is linked to cell acidification during activation of K^+ conductance

Cells were superfused in Hepes-buffered solution containing 10 mM NaSCN (to accelerate AE turnover) and 250 μM CaCl_2. A, confocal imaging under superfusion (at 37°C). (a), addition of 1.5 μM ionomycin activated Ca^{2+}-sensitive K^+ channels, which triggered cell dehydration (due to KCl and water loss), as measured from the SNARF-fluorescence density (interpolated to the isosbestic wavelength of 620 nm; lower panel) and mean cell diameter (grey trace, upper panel). This was accompanied by cell acidification, as measured from the calibrated SNARF-1 ratio (black trace, upper panel). (b), a similar observation was made using 10 μM NS309, a drug that sensitises Ca^{2+}-sensitive K^+ channels to open at resting [Ca^{2+}]_i. B, the response to ionomycin or NS309 was blocked by 300 μM DIDS, the AE inhibitor, implying a central role of AE in mediating the dehydration/acidification response. Each time course is the average of >20 cells. C, flow cytometry analysis of cell volume and pH_i (at 25°C) in cells equilibrated in control medium (with SCN^- and Ca^{2+}) or treated with ionomycin (1.5 μM), NS309 (10 μM) or NS309 with vanadate (1 mM; to raise [Ca^{2+}]_i). Flow cytometry confirms the link between pH_i and volume.
in Hb oxygenation as all media were in equilibrium with atmospheric air. The reason for the lower pH in Hepes buffer was not investigated further, but may be related to the presence of membrane-impermeant, anionic Hepes or a greater degree of haemoglobin ionisation in the absence of intracellular HCO$_3^-$ and/or CO$_2$. The low c.v. for the resting pH frequency distribution (1%; see Fig. 2) indicates a high degree of pH homogeneity among cells (measured with confocal imaging at 37°C and flow cytometry at 25°C). This homogeneity persists when pH$_i$ is displaced from its normal value of 7.4, causing pH$_i$ to settle at a new level, but still with a low c.v. Measurements of average pH$_i$ within a suspended population of RBCs have been reported previously (mean pH$_i$ ∼7.10 at pH$_o$, 7.4), using intracellular BCECF fluorescence in nominally CO$_2$/HCO$_3^-$-free conditions (Kummerow et al. 2000). In that work, however, pH$_i$ estimates were not based on individual RBC measurements, and the in situ nigericin calibration was not validated against an independent (null-point) method so that, as emphasised below, possible measurement errors cannot be excluded.

**SNARF-1: suitable for pH measurement in RBCs, but only with null-point calibration**

The high quantum yield of AM-loaded SNARF-1 in the RBC appears to overcome the quenching power of intracellular haemoglobin, which has been shown to attenuate the fluorescence signal from other ion-sensitive fluorophores, such as the Ca$^{2+}$ indicators Fura-2 and Indo-1 (Lew et al. 1993; Kaestner et al. 2006). Intracellular loading of SNARF-1 also has no effect on resting RBC volume (Supplemental Fig. S1), suggesting that the dye does not disrupt Ca$^{2+}$ and cell volume homeostasis. SNARF-1 may thus be preferable to BCECF for pH$_i$ measurement, as the latter fluorophore is believed to inhibit the plasma membrane Ca$^{2+}$ pump (Gatto & Milanick, 1993).

The SNARF-1 ratio in RBCs was calibrated using two independent approaches (Fig. 1). The null-point approach produced a pH$_i$ calibration curve that was acid-shifted relative to the more commonly used nigericin method (Fig. 1C). Due to the presence of intracellular haemoglobin, [Cl$^-$]$_i$ < [Cl$^-$]$_o$ and therefore pH$_i$ is expected to be more acidic than pH$_o$ when AE1 is at equilibrium. The null-point calibration produced an estimate of RBC resting pH$_i$ (7.15–7.25) that was in agreement with this equilibrium condition, whereas the nigericin method erroneously suggested parity between resting pH$_i$ and pH$_o$. A similar discrepancy between nigericin and null-point calibrations has been reported previously for renal mesangial cells (Boyarsky et al. 1996), but not for other cell types, such as carotid body glomus cells and cardiac myocytes (Buckler & Vaughan-Jones, 1990; Leem et al. 1999), or in the three epithelial cell-lines tested in the present work (Supplemental Fig. S3).

The reason for the calibration discrepancy in RBCs is not clear and requires further investigation. However, it is noteworthy that a significant fraction of RBCs was observed to haemolyse spontaneously in the presence of nigericin. Cells that remain intact under these conditions and provided data for the nigericin calibration curve may therefore represent a subset of cells that is not representative of the whole population. Another possibility is that, when exposed to nigericin, these RBCs do not reach a true steady-state in terms of cell volume and ionic composition. In such a quasi-stable state, transmembrane K$^+$ and H$^+$ gradients may be unequal, thus biasing the nigericin curve. The similarity between nigericin calibrations of RBC pH$_i$ obtained with 100 mM and 140 mM K$^+$, suggests that the error does not simply arise as a result of inappropriate matching of extracellular to intracellular [K$^+$], but may involve factors that are unique to RBCs, such as direct nigericin effects on membrane stability. Significant haemolysis did not occur in RBCs during null-point calibration, and resting pH$_i$ values so derived appeared to be accurate, given that experimental measurements of intracellular CO$_2$/HCO$_3^-$-dependent buffering agreed quantitatively with theoretical predictions, a result that would not occur if estimates of pH$_i$ were in error (see also Boyarsky et al. 1996 for a discussion of this point). When quantifying pH$_i$ in the RBC, the nigericin technique for in situ pH fluorophore calibration should thus be abandoned in favour of the null-point method.

**Intracellular H$^+$-buffering in human RBCs**

The ability to record pH$_i$ permitted the application of an extracellular ammonium-withdrawal technique for estimating buffering capacity directly within the RBC, as illustrated in Fig. 4 (Boyarsky et al. 1988). The resulting value for intrinsic (non-CO$_2$/HCO$_3^-$-dependent) buffering capacity (in excess of 100 mmol (l cell water)$^{-1}$ (pH unit)$^{-1}$) is among the highest observed in any cell type. This is consistent with the high concentration of intracellular haemoglobin, a high-capacity pH buffer (the mean Hb concentration of 5.2 mmoles per litre of packed cells, is equivalent to 7.0 mmol (l cell water)$^{-1}$, assuming that 25% of the cell volume is occupied by Hb molecules) (Lew et al. 1991). Intracellular Hb buffering capacity has previously been estimated from acid-titration of cell-lysates to be between 53 and 68 mmol (l cell)$^{-1}$ (pH unit)$^{-1}$ (equivalent to 70–91 mmol (l cell water)$^{-1}$ (pH unit)$^{-1}$; (Cass & Dalmark, 1973; Dalmark, 1975). The present in situ estimate of intrinsic buffering is thus at least 11% higher than previous indirect estimates, and possibly up to 50% higher, depending on the pH$_i$ at
which β-values are selected (Fig. 4). The discrepancy may indicate the presence of intracellular RBC buffers in addition to haemoglobin, or may point to previous errors in estimates of the proportion of cell volume occupied by water. Alternatively, the assumptions involved in the ammonium-withdrawal method for estimating β may not apply in RBCs.

Measurements of buffering capacity in the presence of physiological CO₂/HCO₃⁻ buffer reveal an additional component of RBC buffering, derived from intracellular CO₂/HCO₃⁻ (Fig. 4B), which has not previously been quantified. The ability of CO₂/HCO₃⁻ to buffer pHᵢ changes is limited by the kinetics of the chemical reaction CO₂ + H₂O ⇌ HCO₃⁻ + H⁺. High intrinsic buffering due to intracellular Hb will tend to slow equilibration of this reaction, and therefore sufficient catalysis by the high levels of intracellular CA is required to enable CO₂/HCO₃⁻ buffering to operate efficiently (Meldrum & Roughton, 1933; Maren, 1967). The present measurements show that intracellular CO₂/HCO₃⁻ can contribute up to a third of total buffering power within the RBC over the physiological pHᵢ range. The close agreement between experimentally derived values for this component of buffering and the theoretical values (predicted to be 2.303 x [HCO₃⁻] in an intracellular compartment fully open to extracellular CO₂; see Fig. 4B and Roos & Boron, 1981) suggests that the ammonium-withdrawal technique for measuring RBC buffering capacity is accurate.

**pHᵢ regulation in human RBCs**

The present work shows that acute displacement of pHᵢ in the acid or alkaline direction is compensated within a few minutes by acid or base extrusion from the cell. The system superficially resembles that observed in most eukaryotic cells. In the case of the RBC, however, the principal pHᵢ sensor and transport effectors are subsumed within a single type of membrane protein, the AE1 Cl⁻/HCO₃⁻ exchanger.

In eukaryotic cells, major acid and base effluxes are typically mediated via separate membrane transport proteins, such as Na⁺/H⁺ exchange (H⁺ efflux) and Cl⁻/HCO₃⁻ exchange (H⁺-equivalent influx) (Boron, 2004; Vaughan-Jones et al. 2009). NHE1 transcripts have been reported in immature RBCs (reticulocytes that comprise ~1% of an RBC population) (Sarangarajan et al. 1998), and an amiloride-sensitive 22Na⁺ uptake has been measured in human RBCs (Escobales & Canessa, 1986). In the present work, however, exposure of RBCs to the NHE inhibitor, dimethyl amiloride, had no effect on pHᵢ recovery from an acid load, while exposure to DIDS, an AE1 inhibitor with no potency against NHE1, blocked acid extrusion completely (Fig. 3Ca). While these results do not exclude the appearance of significant NHE1 activity in certain clinical conditions, like hyperaldosteronism (Koren et al. 1998), they indicate little or no role for the transporter in the regulation of pHᵢ in normal mature RBCs. AE1 is a product of the SLC4 gene family of bicarbonate transporters (SLC4A1). A second gene family (SLC26) also codes for anion exchange transport proteins (Sterling & Casey, 2002; Mount & Romero, 2004), but its products have not so far been identified in RBCs, so that AE1 remains the only candidate HCO₃⁻ transporter. The human mature RBC is thus unusual in that all aspects of transmembrane pHᵢ regulation seem to be accomplished by AE1. As discussed below, this arrangement works successfully for two reasons: (i) AE1 is at ionic equilibrium at resting pHᵢ (i.e. [HCO₃⁻]o/[HCO₃⁻]ᵢ, [Cl⁻]o/[Cl⁻]ᵢ = [H⁺]/[H⁺]o), and (ii) AE1 activity is not allosterically modulated by pHᵢ.

In other cell types, transporters such as NHE and Cl⁻/HCO₃⁻ exchange are typically far from ionic equilibrium at resting pHᵢ, and so are constrained thermodynamically to produce only H⁺-equivalent efflux or influx in the physiological pHᵢ range. In contrast, because it is normally at ionic equilibrium, AE1 in the RBC can operate reversibly, producing acid influx or efflux (Fig. 5A).

The activity of NHE and Cl⁻/HCO₃⁻ exchangers in non-erythroid cells is typically modulated allosterically by H⁺ ions, so that acid/base transport is high only over a narrowly defined range of pHᵢ, sometimes resulting in a preferred polarity for H⁺-equivalent flux across the membrane i.e. flux rectification (Aronson et al. 1982; Olsnes et al. 1986; Leem et al. 1999). In contrast, the present results in the RBC provide no evidence for allosteric pHᵢ modulation of AE1, so that significant activity is observed at both acid and alkaline pHᵢ (Fig. 5A). A lack of allosteric restraint also helps to explain the high dependence of steady-state pHᵢ in the RBC on changes of pHᵢo, a relationship defined by H⁺-equivalent (HCO₃⁻) flux through the AE1 transporter (Fig. 2Bb). The transfer function (ΔpHᵢ) is the steepest measured for any cell type. For example, a typical pHᵢ/pHᵢo slope of ~0.4 has been reported in cardiac cells (Ellis & Thomas, 1976; Sun et al. 1996) while in Xenopus oocytes (Humphreys et al. 1994) it is 0.14. The equilibrium value of pHᵢ will also be responsive to changes in [Cl⁻]o and [Cl⁻]ᵢ (Fig. 6). Consequently, any changes to the net ionisation state of haemoglobin, such as those arising from changes in oxygenation status (Tiffert et al. 1993), will alter pHᵢ via changes in [Cl⁻]o.

The present work shows that AE1 transport activity in the RBC correlates linearly with [HCO₃⁻]o, the concentration of transported substrate (Fig. 5C). As a result, the relationship between pHᵢ and JHᵢ is also approximately linear (Fig. 5A). The pHᵢ sensitivity of AE1 activity is thus likely to be more apparent than
real, reflecting instead a first-order dependence on [HCO$_3^-$]$_i$. This conclusion is consistent with the broad pH insensitivity of $^{36}$Cl$^-$ flux previously reported for AE1 in red cell ghosts (Funder & Wieth, 1966), and in heterologous expression systems (Zhang et al. 1996). AE1 is likely to regulate RBC pH$_i$ by sensing an associated rise or fall of [HCO$_3^-$]$_i$ (via its intracellular anion binding site), and adjusting HCO$_3^-$ transport accordingly. These kinetic properties are very different from other anion exchange isoforms of the SLC4 gene family, such as AE2 and AE3, which are allosterically switched down at low pH$_i$ (Lee et al. 1991; Stewart et al. 2004). The present results therefore suggest that pH$_i$ regulation in the RBC may be the beneficial consequence of a system evolved to regulate [HCO$_3^-$]$_i$. This is perhaps to be expected as AE1’s prime role is to translocate HCO$_3^-$ in either direction across the RBC membrane during the mass transport of CO$_2$ from respiring tissue to lungs. HCO$_3^-$ flux reversal during this cycle is aided by a transporter close to equilibrium at resting pH$_i$, and without allosteric restraint. This design, nevertheless, means that RBC pH$_i$ can, when required, be efficiently controlled, as shown in the present work. This is desirable, as the RBC’s protein machinery, including its Hb and its glycolytic and CA enzymes are all H$^+$ sensitive, with a proscribed pH$_i$ range for optimal function.

DIDS-sensitive pH$_i$ regulation in the RBC is still evident in the nominal absence of CO$_2$/HCO$_3^-$ (Fig. 3Aa, B and C), albeit at a much slower rate (Fig. 3Ab), raising the possibility that OH$^-$ may be transported as well as HCO$_3^-$, particularly when levels of the latter are low. While this occurs for H$^+$-equivalent transport on certain SLC26 gene products (Alvarez et al. 2004, but see Knauf et al. 2001), kinetic analyses have suggested that AE1, an SLC4A1 gene product, does not transport OH$^-$, but operates instead on residual HCO$_3^-$ levels (Papageorgiou et al. 2001; Knauf et al. 2002). Because of the presence of atmospheric CO$_2$, intracellular and extracellular [HCO$_3^-$] are constitutively in the range 100–350 μM (and will vary with pH$_i$), which appears to be sufficient to support a low level of AE1 activity (Knauf et al. 2002). Rapid pH$_i$ regulation in the RBC is thus likely to be dependent on the transport of HCO$_3^-$, and regulated by its ambient concentration.

**pH$_i$–volume coupling in human RBCs**

Although pH$_i$ is tightly regulated, it is nevertheless modulated by changes of RBC volume. In some cases the volume change may be driven directly through AE1 activity. For example, raising pH$_o$ (at constant CO$_2$ partial pressure) induces a modest cell shrinkage by driving H$^+$ and Cl$^-$ efflux through AE1 (equivalent to Cl$^-$ efflux/HCO$_3^-$ influx), which raises pH$_i$ (Fig. 7). Cell dehydration occurs (Fig. 7) because the ensuing [Cl$^-$]$_i$ reduction is not balanced by an osmotically equivalent rise of [HCO$_3^-$]$_i$ (owing to intracellular H$^+$-buffering). The primary activation of cell dehydration via AE1 is thus linked directly with intracellular alkalosis, as illustrated schematically in Fig. 9A. Another example of this type of pH$_i$–volume coupling would be activating AE1 by reducing [Cl$^-$]$_o$ at constant extracellular osmolarity (replacing extracellular Cl$^-$ with a membrane-impermeant anion). As shown in Fig. 6, this raises pH$_i$ as Cl$^-$ exits from the RBC in exchange for HCO$_3^-$ via AE1. The reaction between intracellular HCO$_3^-$ and H$^+$ ions yields CO$_2$ which exits cells freely. Consequently, Cl$^-$ efflux produces a net loss of osmolyte, which is accompanied by water loss and cell shrinkage (Lew & Bookchin, 1986).

In contrast to the effects of directly activating AE1, induction of KCl efflux through the opening of KCNN4 (KCa3.1) Gardos channels in the surface membrane is a powerful primary stimulus for cell dehydration, directly
inducing an osmotic efflux of water. By reducing [Cl\(^-\)], the KCl loss disturbs the AE1 transporter’s equilibrium state, resulting in a Cl\(^-\) influx/HCO\(_3\)\(^-\) efflux, as predicted by the RBC model (Lew & Bookchin, 1986). The cell thus acidifies, as illustrated in Fig. 9B (a more detailed schematic representation of the underlying ionic changes is available in Supplemental Fig. S4). In this case, the primary activation of cell dehydration through KCl efflux then induces a secondary acidosis through AE1. Thus, manoeuvres that alter RBC volume also induce a change of pH\(_i\). But depending on whether Cl\(^-\)/HCO\(_3\)\(^-\) exchange is the primary cause of the volume change (Fig. 9A), or is activated secondarily (Fig. 9B), the pH\(_i\) signals move in opposite directions. Nevertheless, through recruitment of AE1 activity, volume changes in the RBC are inevitably coupled to pH\(_i\) changes. In effect, the equilibrium condition for pH\(_i\) regulation by AE1 is reset to a new resting pH\(_i\) by a volume disturbance.

Under physiological conditions, Ca\(^{2+}\)-activated KCNN4 channels are silent, except perhaps in ageing cells with slightly elevated [Ca\(^{2+}\)], where they may be minimally activated, contributing to the homeostatic changes observed during RBC senescence (Lew et al. 2007; Tiffert et al. 2007). In certain diseased states, such as sickle-cell anaemia, the channels play a key role in disease pathogenesis, and their activation leads to the formation of a subpopulation of young, hyperdense and irreversibly sickled red cells (Lew & Bookchin, 2005). The combination of cell dehydration and cell acidification was proposed as a critical link in the generation of these hyperdense cells (Lew et al. 1991). Measurements of extracellular pH have indirectly supported the existence of this volume–pH\(_i\) coupling and an involvement of anion exchange (Freeman et al. 1987). Mathematical modelling of RBC ion homeostasis also predicts volume–pH\(_i\) coupling. The present work now provides the first direct measurement of the coupling, and confirms the central role of AE1.

Conclusions

Imaging pH\(_i\) confocally in individual RBCs, as well as profiling it in large populations using flow cytometry, provides a new tool for exploring hydrogen ion dynamics in erythrocytes, in both health and disease. As with other cells, an efficient control of pH\(_i\) in the RBC is necessary for the maintenance of normal activity. Furthermore, the functional coupling between volume and pH\(_i\) recorded in the present work need not be exclusive to the erythrocyte. It may be applicable to any cell type that expresses Cl\(^-\)/HCO\(_3\)\(^-\) exchange at the surface membrane (including products of the SLC26 as well as the SLC4 gene families). The amplitude of the volume-associated pH\(_i\) signal will depend, in part, on the kinetic properties of the anion exchanger, as well as on the co-expression and functional activity of other plasmalemmal acid/base extruders. In some cells, a functional coupling between anion exchange and Na\(^+\)/H\(^+\) exchange results in pH\(_i\)-neutral volume regulation (Mason et al. 1989). Nevertheless, the principle that the anion exchanger can also couple changes of cell volume to a significant pH\(_i\) signal should not be ignored when considering the integrated response of a cell.

Appendix

The null-point method for calibrating pH sensitive dyes has been discussed in detail elsewhere (Eisner et al. 1989; Buckler & Vaughan-Jones, 1990). The method assumes that cell entry of the charged form of weak acid/base (acetate Ac\(^-\), ammonium NH\(_4\)\(^+\)) is negligible over the time period tested. This is supported by pH\(_i\) time course data in the presence of DIDS (Fig. 3). Exposure to ammonium-containing solution (Fig. 3A) produces an intracellular alkalinisation (NH\(_3\) entry) with no secondary acidification (i.e. no NH\(_4\)\(^+\) entry). Similarly, exposure to acetate-containing solution (Fig. 3B) produces acidification (acetic acid entry) but no secondary alkalinisation. A certain combination of weak acid (acetate/acyclic acid) and weak base (ammonium/ammonia) produces no net change in pH\(_i\) at steady-state. At this pH\(_i\), [NH\(_4\)\(^+\)] = [Ac\(^-\)]. Assuming that weak acid/base ionisation constants are the same on either side of the membrane, it is possible to derive a relationship linking pH\(_o\), the concentration of weak acid (T\(_A\) = [Ac\(^-\)]\(_o\) + [HAc\(_o\)]), weak base (T\(_B\) = [NH\(_3\)]\(_o\) + [NH\(_4\)\(^+\)]\(_o\)) and the null point pH\(_i\) (pH\(_{null}\)):

$$\sqrt{\frac{T_B}{T_A}} = \frac{[H^+]_o}{[H^+]_i} = 10^{pH_{null} - pH_i}$$

When no change in steady-state dye fluorescence is observed in a cell superfused with weak acid and base, in a ratio equal to T\(_B\)/T\(_A\), resting pH\(_i\) (i.e. pH\(_{null}\)) can be derived from the equation above. The null-point calibration time courses produce initial, transient alkaline displacements of pH\(_i\), before attaining a steady-state pH\(_i\), because NH\(_3\) is more membrane permeant than the large HAc molecule. However, the derivation above relates to the steady-state condition, once NH\(_3\) and HAc have attained equal transmembrane distribution.

References


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Author contributions

experiments (confocal imaging) in DCEB, Cambridge. All authors have approved the final version for publication.

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